Evaluation of Protein Glycation and Antioxidant Levels in Birds of Prey

by

Tana Ingram

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Graduate Supervisory Committee:

Karen Sweazea, Chair
Carol Johnston
Christy Lespron

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ABSTRACT

Birds have shown promise as models of diabetes due to health and longevity despite naturally high plasma glucose concentrations, a condition which in diabetic humans leads to protein glycation and various complications. Research into mechanisms that protect birds from high plasma glucose have shown that some species of birds have naturally low levels of protein glycation. Some hypothesize a diet rich in carotenoids and other antioxidants protects birds from protein glycation and oxidative damage. There is little research, however, into the amount of protein glycation in birds of prey, which consume a high protein, high fat diet. No studies have examined the potential link between the diet of carnivorous birds and protein glycation. The overall purpose of this study was to evaluate whether birds of prey have higher protein glycation given their high protein, high fat diet in comparison to chickens, which consume a diet higher in carbohydrates. This was accomplished through analyses of serum samples from select birds of prey (bald eagle, red-tailed hawk, barred owl, great horned owl). Serum samples were obtained from The Raptor Center at the University of Minnesota where the birds of prey consumed high protein, high fat, non-supplemented diets that consisted of small animals and very little to no carbohydrate. Serum was also obtained from one chicken for a control, which consumed a higher carbohydrate and antioxidant-rich diet. Glucose, native albumin glycation and antioxidant concentrations (uric acid, vitamin E, retinol and several carotenoids) of each sample was measured. Statistical analyses showed significant between group differences in percent protein glycation amongst the birds of prey species. Glycation was significantly higher ($p < 0.001$) in bald eagles ($23.67 \pm 1.90\%$) and barred owls ($24.28 \pm 1.43\%$) compared to red-tailed hawks ($14.31 \pm 0.63\%$).

Percent glycation was higher in all birds of prey compared to the chicken sample and literature values for chicken albumin glycation. Levels of the carotenoid lutein were significantly higher in bald eagles and barred owls compared to great horned owls and red-tailed hawks and the carotenoids beta-cryptoxanthin and beta-carotene were significantly greater in bald eagles compared to red-tailed hawks and great horned owls.

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INTRODUCTION

Background. For the last several decades, worldwide diabetes mellitus prevalence has increased. In the United States alone in 2011-2012, it was estimated that 12-14% of adults had diabetes (both diagnosed and undiagnosed) while another 37-38% had prediabetes, the metabolic precursor to diabetes, bringing the total estimated number of people with either diabetes or prediabetes to 49-52% of the overall population (Menke, 2015). With such a high prevalence, it is not surprising diabetes is a major cause of morbidity and mortality in the United States and costs the nation upwards of $245 billion annually in healthcare costs and loss of productivity (Menke, 2015; American Diabetes Association, 2013). Globally in 2013, diabetes and diabetes-related complications cost $549 billion or 11% of worldwide healthcare expenditures (International Diabetes Federation, 2013). It is projected the prevalence of type 2 diabetes in those under 20 years old will quadruple in 40 years and, by 2040, approximately 592 million people worldwide will have diabetes (American Diabetes Association, 2014; International Diabetes Federation, 2013). These statistics highlight the need for continued research into the prevention and treatment of diabetes and diabetes-related complications.

Hyperglycemia is the hallmark of diabetes. Chronically high levels of plasma glucose can cause microvascular damage of the retinas, kidneys and peripheral nerves leading to blindness, kidney disease and various neuropathies (Brownlee, 2001). In addition to these complications, hyperglycemia damages arteries, accelerates atherosclerosis and increases the risk of myocardial infarction, stroke, and limb amputation (Brownlee, 2001; Basta 2004). Micro- and macrovascular damage can be

caused by hyperglycemia-mediated glycation of plasma proteins, which then slowly undergo a series of reactions to form advanced glycation end products (AGEs) (Basta, 2004). AGEs can form reactive oxygen species (ROS) and are implicated in the development of diabetic complications (Korwar et al., 2015; Brownlee, 2001; Basta, 2004). AGEs accumulate in tissues and because of their stability, levels do not decrease even after hyperglycemia is corrected (Makita et al., 1991). This means the current treatment for diabetes, controlling plasma glucose levels, may not be enough to prevent further complications of the disease, due to AGE accumulation in the body that occurred before diagnosis and/or treatment. For this reason, further research into the prevention of protein glycation and AGE formation is needed.

To fully understand the role of protein glycation in the pathogenesis of diabetic complications, appropriate animal models are needed. Due to the slow progression of the disease, during which time it can take years for symptoms of complications to manifest, conventional animal models, such as short-lived mice and rats, do not provide data that is comparable to humans (Szwergold & Miller, 2013). Longer-lived animals, such as monkeys and pigs, can pose logistical research problems that make long-term study both difficult and expensive (Szwergold & Miller, 2013). Birds as a class have been suggested as a fitting model for diabetes research. While in the past birds have been used to study aging, due to certain biochemical characteristics it has been proposed birds would also provide a useful model of diabetes (Szwergold & Miller, 2013). In fact, despite having higher metabolic rates, body temperatures and plasma glucose concentrations than mammals, birds are models of healthy aging as they outlive mammals of comparable body size by up to three times (Braun & Sweazea, 2008; Holmes, et al., 2001). Despite

these characteristics, birds are free of the pathologies that would arise in humans under the same circumstances. Discovering what protects birds from the deleterious consequences of naturally high plasma glucose concentrations would aid in creating new approaches to preventing and treating diabetes and its complications. Specifically, research into how birds prevent high levels of protein glycation despite their naturally high plasma glucose concentrations is integral to developing novel diabetes prevention and treatment strategies.

Studies have found that despite having high plasma glucose concentrations, birds are resistant to the glucose-lowering effects of insulin (For review: Braun & Sweazea, 2008) and have low levels of protein glycation (Holmes, et al., 2001). However, because many of the studies to date have used domestic species such as chickens, it is not known if the same is true for all species or wild birds. As granivores, chickens consume mainly grains and seeds. Thus, their overall diet is low in fat and protein (O'Donnell, 1978) and higher in carbohydrates and antioxidants. Birds of prey, on the other hand, consume a diet high in protein and fat. Intriguingly, some birds of prey, such as great horned owls and red-tailed hawks, have higher plasma glucose concentrations than chickens, which is attributed to higher rates of gluconeogenesis to compensate for low dietary carbohydrate intake (O'Donnell, 1978). Thus diet may be responsible for the difference between the birds of prey and chickens in this regard (O'Donnell, 1978). To our knowledge, no studies have examined a potential link between diet and protein glycation in birds. Research of this sort would be of interest to humans because it is widely accepted that diet and lifestyle contribute to the development and exacerbation of type 2 diabetes (International Diabetes Federation, 2013). An understanding of how specific nutrients

affect protein glycation in the presence of high plasma glucose concentrations is essential to discovering new therapies for humans in the prevention of complications that arise from protein glycation and AGEs in the course of diabetes.

Purpose of Study. The overall purpose of this research study was to evaluate whether birds of prey have higher protein glycation given their high protein, high fat diet in comparison to chickens which consume a diet higher in carbohydrates. This will be accomplished through analyses of serum samples from select birds of prey (bald eagle, red-tailed hawk, barred owl, great horned owl).

Research Aim and Hypotheses. The main research aim of this study was to evaluate native protein glycation in several birds of prey in comparison to chickens.

H1: Native protein glycation will be higher in birds of prey as compared to chickens due to their high protein, high fat diet with limited ingestion of antioxidants.

H2: Uric acid levels will be higher in birds of prey than chickens due to higher ingestion and utilization of protein for energy.

H3: Carotenoid and antioxidant levels will be lower in birds of prey than chickens due to low ingestion of antioxidants.

Definition of Terms. The following is a list of terms and the author's definition of terms that are commonly used throughout this thesis.

AGEs – advanced glycation end products; irreversible compounds that form in the presence of hyperglycemia and promote the complications associated with diabetes.

Birds of prey – a predatory bird, distinguished by a hooked beak and sharp

talons.

Carnivore – an animal that eats a diet which consists mainly of the flesh and tissues of other animals, which is high in protein and fat.

DAG-PKC pathway – during hyperglycemia, increased diacylglycerol (DAG) from glycolysis activates protein kinase C (PKC) isoforms, which can lead to retinal and renal blood flow abnormalities, decreased production of nitric oxide in the kidneys, endothelial dysfunction, vascular permeability, changes in vessel dilation and basement membrane thickening.

GLUT-4 – the predominant insulin-responsive glucose transporter in mammals, which translocates from the intracellular space to the cell wall to facilitate glucose uptake from the blood in response to insulin binding to cell receptors.

Glucose auto-oxidation – *in vitro*, glucose is prone to transition metal-catalyzed oxidation which generates reactive oxygen species that in turn promote and contribute to overall protein glycation.

Glycation – non-enzymatic addition of sugars to proteins, lipoproteins and nucleic acids.

Granivore – a type of herbivore that primarily eat seeds and grains and therefore, consumes a relatively high carbohydrate, low protein, low fat diet.

Hexosamine pathway (HSP) – plays a small role in normal glucose metabolism; use is increased during hyperglycemia.

Hyperglycemia – an excess of glucose in the bloodstream

Insulin resistance – condition in which the cells of the body become resistant to the effects of the hormone insulin, thus causing glucose to build up in the bloodstream.

Neuropathy – nerve damage that causes weakness or pain in affected area.

Nephropathy – kidney damage or disease.

Polyol pathway – pathway in which glucose is reduced to sorbitol and converted to fructose.

Prediabetes – the metabolic precursor to diabetes defined as having a hemoglobin A_{1c} between 5.7-6.4%, fasting plasma glucose between 100-125 mg/dL or two-hour glucose during an oral glucose tolerance test (OGTT) between 140-199 mg/dL.

Protein kinase C (PKC) – a group of enzymes important in many cellular functions and signal transduction pathways.

RAGE – receptors for AGEs; a molecule belonging to the immunoglobulin superfamily that has multiple ligands, including AGEs.

Reactive oxygen species (ROS) – highly reactive molecules, which in excess quantities, can damage cells and tissues and lead to the loss of physiological function.

Retinopathy – pathological damage to the retina of the eye.

Delimitations. Serum samples for specific birds of prey were used, thus the results may not be extrapolated to the whole animal or other birds, animals or humans.

Limitations. Some limitations of this study would be the use of serum from captive birds as opposed to wild birds and not manipulating the diet of the birds to determine a direct link between dietary modifications and glycation. Also, while the diet of the birds of prey consisted of small animals, it may not be representative of the type and predictability of feedings that birds of prey may experience in the wild. Because of inherent physiological differences between mammals and birds, findings from this study may not directly influence recommendations for humans, but may be used as a guide for further research and investigation.

CHAPTER 2

REVIEW OF LITERATURE

I. Diabetes Mellitus.

Prevalence. For the last several decades, worldwide diabetes mellitus prevalence has increased. It is projected by the year 2035, approximately 592 million people worldwide will have or be affected by diabetes (International Diabetes Federation, 2013). The rate of diabetes has increased over time in every age group, in both genders, in every racial and ethnic group, as well as across all socioeconomic and education levels (Menke et al., 2015). It was estimated in the year 2013 alone, that an individual died from diabetes or its related complications every six seconds, totaling 5.1 million people (International Diabetes Federation, 2013). In the same year, diabetes and diabetes-related complications cost $549 billion or 11% of worldwide healthcare spending (International Diabetes Federation, 2013).

In the United States in 2011-2012, it was estimated that 12-14% of adults had diabetes (both diagnosed and undiagnosed), while another 37-38% had prediabetes, the metabolic precursor to diabetes, bringing the total estimated number of people with either diabetes or prediabetes to 49-52% of the overall population (Menke, 2015). With such a high prevalence, it is not surprising diabetes is a seventh leading cause of morbidity and mortality among adults in the United States (National Center for Health Statistics, 2015) and costs the nation upwards of $245 billion in healthcare costs and loss of productivity (Menke, 2015; American Diabetes Association, 2013).

People of all ages can be affected by diabetes although the majority of those diagnosed with diabetes are between the ages of 40 and 59 (International Diabetes

Federation, 2013). However, the prevalence of type 1 diabetes is increasing at a rate unexplained by genetic factors (Ramasamy et al., 2011; American Diabetes Association, 2014); in 2013, more than 79,000 children had type 1 diabetes (International Diabetes Federation, 2013). In addition, the incidence of type 2 diabetes in adolescents is increasing, especially among those in minority groups (American Diabetes Association, 2013). It is projected the prevalence of type 2 diabetes in those under 20 years old will quadruple in 40 years (American Diabetes Association, 2014). This is troubling because research shows individuals diagnosed with type 2 diabetes at a young age (15-30 years old) have greater rates of morbidity and mortality than those diagnosed in middle age (Al-Saeed et al., 2016). This increase in the early onset of diabetes in young people is alarming and highlights the need for continued research into the prevention and treatment of diabetes and diabetes-related complications for people of all ages.

Pathology. Diabetes is a disturbance in glucose regulation with hyperglycemia as its hallmark characteristic. In healthy individuals, glucose is regulated by the counterregulatory hormones insulin and glucagon. Insulin is produced by the beta cells of the islets of Langerhans in the endocrine pancreas; insulin enables glucose uptake into cells of insulin-dependent tissues, such as muscle and adipose, and is important in carbohydrate, lipid and protein metabolism (Wilcox, 2005) Insulin is mitogenic, meaning it supports and promotes cell division and growth and thus directs the anabolic processes of the fed state (Wilcox, 2005). Insulin deficiency, or resistance to its effects, leads to chronic hyperglycemia, which leads to the development of diabetes and diabetic complications.

Diabetes is classified into four categories depending on the pathogenesis of chronic hyperglycemia according to the American Diabetes Association. The four categories are type 1 diabetes, type 2 diabetes, gestational diabetes mellitus (GDM), and 'other', which includes diabetes due to genetic defects, diseases of the exocrine pancreas, and drug or chemical induced diabetes (American Diabetes Association, 2014).

Type 1 diabetes. Hyperglycemia of type 1 diabetes is precipitated by an autoimmune destruction of the beta cells in the pancreas, which leads to insulin deficiency (American Diabetes Association, 2014). Approximately 5% of all diabetes cases in the United States are type 1 diabetes (Centers for Disease Control and Prevention, 2014). There is a strong genetic factor in the development of diabetes; in twin studies, if one of the twins developed type 1 diabetes, the other twin had a ~50% chance of developing the disease (Hyttinen et al., 2003). It is thought the destruction of beta cells is autoimmune-mediated because patients with type 1 diabetes typically have antibodies to islet cells (Decochez et al., 2000). In fact, many studies have suggested measuring islet autoantibodies in relatives of those diagnosed with type 1 diabetes to determine if they too are at risk for developing the disease, since detection and diagnosis may limit complications and extend the time the pancreas produces insulin (American Diabetes Association, 2014). The onset of type 1 diabetes usually occurs in those younger than 20 years old and is typically sudden, with individuals experiencing elevated levels of plasma glucose and often times, life-threatening ketoacidosis resulting from fatty acid oxidation in lieu of glucose (American Diabetes Association, 2014). Individuals with type 1 diabetes require life-long treatment with exogenous insulin in order to control and

maintain safe levels of plasma glucose (Fullerton, et al., 2014). People with type 1 diabetes will die without insulin (International Diabetes Federation, 2013).

Type 2 diabetes. This form of diabetes develops as a response to chronic insulin resistance and a progressive decrease in insulin production due to the resistance (Wilcox, 2005). When plasma glucose levels are high, insulin is released from the beta cells of the islets of Langerhans in the pancreas into the bloodstream. One of the major roles of insulin is to facilitate the uptake of glucose into cells. This happens through increased insulin delivery of glucose to tissues via insulin-mediated vasodilation as well as insulin binding to its receptors in the cell membrane of muscle and adipose tissues where, through a cascade of intracellular signaling events, it stimulates the translocation of the insulin-responsive glucose transporter GLUT-4 to the plasma membrane to facilitate glucose uptake into the cell (Shepard & Kahn, 1999). The importance of GLUT-4 to glucose homeostasis has been shown in muscle-selective and adipose-selective GLUT-4 knockout mice; these mice develop insulin resistance, glucose intolerance and impaired insulin action without GLUT-4 (Seki et al., 2003). Of note, glucose uptake in mammals can occur by two different processes; one is through the insulin-responsive GLUT-4 pathway, another is through contraction-responsive GLUT-4, which is translocated to the cell wall due to muscle contraction (Shepard & Kahn, 1999).

Insulin resistance is thought to be due to post-receptor defects in insulin signaling and glucose transporters (Shepard & Kahn, 1999; Wilcox, 2005); meaning insulin can bind to its receptors, but cell binding does not result in the translocation of GLUT-4. Individuals with type 2 diabetes are initially able to produce sufficient amounts of insulin, however, cellular response to insulin is diminished. Ultimately, insulin resistance results

in hyperglycemia because the cells are unable to take up glucose and thus levels in the blood remain high. Approximately 95% of all cases of diabetes are type 2 diabetes (Centers for Disease Control and Prevention, 2014).

Progression of type 2 diabetes occurs slowly over a number of years and is often asymptomatic (American Diabetes Association, 2014; International Diabetes Federation, 2013). Lifestyle factors such as over-nutrition and physical inactivity are risk factors for insulin resistance (Ramasamy, 2011) and are thought to influence a genetic pre-disposition to the development of type 2 diabetes (Kujala, Kaprio, & Koskenvuo, 2002). Insulin resistance is positively correlated with body mass index, waist circumference, and waist-hip ratio increases (Wilcox, 2005). Moreover, high levels of free fatty acids in the blood from over-nutrition are associated with a decrease in insulin secretion and synthesis (Wilcox, 2005). Ageing, ethnicity, obesity and family history of diabetes are other risk factors for developing type 2 diabetes (International Diabetes Federation, 2013).

Current treatment for those at risk of type 2 diabetes includes diet and lifestyle modifications as well as the use of drugs such as metformin, α-glucosidase inhibitors, orlistat and thiazolidinedione (American Diabetes Association, 2014). Lifestyle modification has been shown to be effective in decreasing the progression of diabetes, while the drugs mentioned decrease symptoms of diabetes to varying degrees (American Diabetes Association, 2014). Lifestyle modification and oral drug use are often enough to control plasma glucose concentrations, but some individuals also require insulin to maintain glucose control (International Diabetes Federation, 2013).

Gestational diabetes mellitus. As the name implies, gestational diabetes occurs during pregnancy. Around the 24th week of pregnancy and throughout the third trimester,

a mother may develop insulin resistance most likely due to hormones from the placenta that are secreted to divert glucose and lipids to the fetus (International Diabetes Federation, 2013; Wilcox, 2005). Having gestational diabetes increases risks for both mother and infant. While gestational diabetes usually disappears after the birth of the child, the mother is at increased risk of preeclampsia and caesarean section, gestational diabetes with later pregnancies and development of type 2 diabetes (International Diabetes Federation, 2013). For the infant, gestational diabetes can lead to a larger than average size, putting the newborn at risk for shoulder injuries and breathing problems at birth (International Diabetes Federation, 2013). In addition, infants born to mothers with gestational diabetes are at increased risk for future obesity, development of type 2 diabetes and other metabolic consequences (American Diabetes Association, 2013). In 2013, it was estimated that globally 21 million births were affected during pregnancy by diabetes (International Diabetes Federation, 2013). Lifestyle modification is usually sufficient to manage hyperglycemia in 80-90% of women with gestational diabetes (American Diabetes Association), while a small number may require insulin or oral anti-hyperglycemic medications (International Diabetes Federation, 2013).

Other diabetes. Specific types of diabetes caused by factors other than immune destruction of beta cells or insulin resistance are relatively rare, comprising ~1-5% of all diabetes cases (Centers for Disease Control and Prevention, 2014). These other causes include genetic defects in either beta cell function or insulin action, and diseases such as cystic fibrosis (American Diabetes Association, 2014). Maturity-onset diabetes of the young (MODY) is an example of how genetic defects can cause diabetes; with this type of diabetes, abnormalities in any of the various genes responsible for insulin secretion can

be pathological (Timsit et al., 2016). Drugs or chemicals used to treat HIV/AIDS or used after organ transplantation can also induce diabetes (American Diabetes Association, 2014).

Diagnosis. Diagnosis of diabetes is based on meeting one of three testing criteria on two separate testing occasions. The three criteria are hemoglobin A_{1c} ($HbgA_{1c}$) ≥ 6.5%, a fasting plasma glucose ≥ 126 mg/dL (7.0 mM/L), and a two-hour glucose during an oral glucose tolerance test (OGTT) ≥ 200 mg/dL (11.1 mM/L) (American Diabetes Association, 2014). Normal levels are HbA_{1c} < 5.7%, a fasting plasma glucose < 100 mg/dL (5.6 mM/L) and a two-hour glucose during an oral glucose tolerance test (OGTT) < 140 mg/dL (7.8 mM/L) (American Diabetes Association, 2014).

For individuals who do not have diabetes but whose glucose levels are too high to be normal, the term "prediabetes" is used. Prediabetes is defined as having a HbA_{1c} between 5.7-6.4%, an impaired fasting plasma glucose between 100-125 mg/dL (5.6-6.9 mM/L) or an impaired two-hour glucose during an oral glucose tolerance test (OGTT) between 140-199 mg/dL (7.8-11.0 mM/l) (American Diabetes Association, 2014). Having prediabetes greatly increases the risk of type 2 diabetes and is also associated with the development of cardiovascular disease (International Diabetes Federation, 2013). Prediabetes is not a clinical diagnosis, but is another risk factor associated with the development of type 2 diabetes and is associated with obesity, dyslipidemia with high triglycerides and/or low HDL, as well as hypertension (American Diabetes Association, 2014).

Complications associated with diabetes. Cardiovascular disease is the leading cause of morbidity and mortality among those with diabetes (American Diabetes

Association, 2014; International Diabetes Federation, 2013). Cardiovascular disease accounts for the majority of direct and indirect costs associated with diabetes (American Diabetes Association, 2014). Hyperglycemia, in combination with insulin resistance, damages the arteries that supply the heart and leads to accelerated atherosclerosis and increased risk of myocardial infarction and stroke (Brownlee, 2001; Basta 2004). Other cardiovascular diseases diabetics are at increased risk of are angina, peripheral arterial disease and congestive heart failure (International Diabetes Federation, 2013). At the microvasculature level, hyperglycemia promotes endothelial dysfunction and inhibits production of nitric oxide, a vasodilator and anti-atherogenic molecule (Brownlee, 2001). Endothelial cells are important in the health of the vasculature; these cells line the blood vessels and influence vessel tone and platelet function (Wilcox, 2005). In addition, hyperglycemia and insulin resistance are involved in the pathogenesis of diabetic dyslipidemia, which increases the likelihood of developing atherosclerosis and its associated risk of cardiovascular disease (Brownlee, 2001). Individuals with diabetes often have comorbidities which increase the risk of cardiovascular disease as well, such as hypertension, dyslipidemia, and family history of coronary disease; however, diabetes is an independent risk factor for cardiovascular disease (American Diabetes Association, 2014).

Chronic hyperglycemia leads to the development of disabling and life-threatening microvascular diseases (Cade, 2008; Brownlee, 2001, International Diabetes Federation, 2013). Diabetes-specific microvascular pathologies can cause damage to the retinas, kidneys, and peripheral nerves and are thus the leading causes of blindness, kidney disease and failure, as well as various neuropathies (Brownlee, 2001). Clinical studies

show a strong relationship between glycemia and microvascular complications in both type 1 and type 2 diabetics (The Diabetes Control and Complications Trial Research Group, 1993) as the mechanisms responsible for the pathologies observed in the eyes, kidneys, and nerves are similar and follow the same pathways (Brownlee, 2001; Cade 2008).

Diabetic retinopathy is the most frequent cause of blindness in adults aged 20-74 years (American Diabetes Association, 2014). Damage occurs to the retina, the macula, or both, and in severe cases, abnormal vessel growth appears (Cade, 2008). Severity of the retinopathy and damage to the eye is directly related to the length of time an individual has had diabetes and the degree of hyperglycemic exposure (American Diabetes Association, 2014; Hammes, 1991). Beyond hyperglycemia, factors that put diabetics at higher risk for retinopathy are hypertension and nephropathy (American Diabetes Association, 2014). Other factors associated with diabetic retinopathy are younger age of diabetes onset, insulin treatment, smoking, abnormal lipid profile and a high fat diet (Cade, 2008). It is estimated retinopathy takes as long as five years to develop from the onset of diabetes, a period in which individuals can be asymptomatic (American Diabetes Association, 2014), but nearly 100% of type 1 and type 2 diabetics are estimated to have pathological retinal changes within 15-30 years of having the disease (Hammes, 1991). Surprisingly, type 1 diabetics taking insulin have the highest prevalence of retinopathy, while type 2 diabetics diagnosed after 30 years of age have the lowest prevalence of retinopathy (Klein et al., 1984). The first marker of retinopathy is the loss of pericytes, cells which wrap around endothelial cells and capillaries and aid in capillary tone, growth and protection from reactive oxygen species (ROS) (Wilkinson-

Berka, 2004). The exact mechanisms that lead to pericyte apoptosis are still being investigated, but oxidative stress, formation of advanced glycation end products (AGEs), activation of protein kinase C (PKC) and increased polyol pathway flux are thought to play a role in the development of retinopathy (Cai & Boulton, 2002). Hyperglycemic conditions promote pericyte loss and basement membrane thickening, thus weakening vessel walls, which allows micro-aneurisms and vascular leakage to occur (Wilkinson-Berka, 2004) resulting in capillary blockage that leads to hypoxia and damage to the retina (Kohner, Patel & Rassam, 1995).

Diabetic nephropathy also arises as a result of microvascular damage and occurs in 20-40% of those with diabetes (American Diabetic Association, 2014). Nephropathy will first manifest as microalbuminuria (elevated levels of albumin in the urine) which progresses to albuminuria, indicating renal dysfunction (Drummond & Mauer, 2002). Hyperglycemia promotes the thickening of glomerular basement membranes and triggers glomerular hyperfiltration which causes changes in the cellular matrix and advances to glomerular sclerosis and renal failure (Friedman, 1990). It has been observed that type 2 diabetics who have microalbuminuria or a more advanced stage of diabetic nephropathy will experience a worsening of their condition at a rate of 2-3% a year (Alder et al., 2003). Diabetic nephropathy is the leading cause of end-stage renal disease and its risk factors are similar to diabetic retinopathy; hyperglycemia, duration and age at onset of diabetes, dyslipidemia, hypertension and obesity (American Diabetes Association, 2014).

Neuropathy, or nerve damage, is another serious complication of diabetes; at least one-half of people with diabetes have some form of neuropathy (Dyck et al., 1993). Nerve damage can manifest in a variety of different neuropathies including distal

symmetric neuropathy, diabetic autonomic neuropathy, cardiovascular autonomic neuropathy, gastrointestinal neuropathy and genitourinary tract disturbances (American Diabetes Association, 2014). This list illustrates how nerve damage can negatively affect numerous organ systems. Symptoms of neuropathy related to sensation include pain, burning and tingling sensations, numbness and loss of sensation, while other symptoms can include tachycardia, orthostatic hypotension, gastroparesis, and impaired neurovascular function (American Diabetes Association, 2014). Distal symmetric neuropathy, specifically, can cause severe and sudden pain and is associated with a lower quality of life, limited mobility, depression and social dysfunction (American Diabetes Association, 2014). Amputation and foot ulcerations are common in diabetic neuropathy due to a loss of lower-extremity sensation and peripheral vascular dysfunction (Boulton, 1997). The risk of ulcers or amputation is increased in individuals who have had a previous amputation, past foot ulcer history, diabetic nephropathy, visual impairment, history of cigarette smoking and poor glycemic control (American Diabetic Association, 2014). Hyperglycemia impairs neuronal microvasculature which is suspected to lead to the demyelination of neurons, hence the alterations in sensation (Cade, 2008). Pathologic traits present in neuropathy include pericyte, microfilament and axonal loss, basement membrane thickening, and decreased blood flow to C fibers, all of which lead to decreased perfusion and hypoxia of the connective tissue that surrounds the myelin sheath of each nerve fiber (Dyck & Giannini, 1996; Ibrahim et al., 1999; Malik et al., 1993; Young et al., 1992). At this time, no specific treatment is available for nerve damage, other than glycemic control which may slow progression of neuropathy, but will not reverse nerve damage already sustained (American Diabetes Association, 2014).

As mentioned, current treatments for diabetes include diet and lifestyle modifications, insulin, and the use of drugs such as metformin, α-glucosidase inhibitors, orlistat and thiazolidinedione. These treatments have the goal of maintaining a safe level of plasma glucose concentrations to halt or delay the complications of diabetes, yet vary in their success of glycemic control. Reviews of the literature have shown that intensive glycemic control leads to better health outcomes and slows the progression of many of the complications associated with this disease (American Diabetes Association, 2014; Fullerton, 2014; Ramasamy, 2011). However, recent studies have found surprising outcomes of intensive glycemic control as intensive treatment of hyperglycemia and the possible risk of severe hypoglycemia may be detrimental and do not result in a reduction of cardiovascular risk (ACCORD Study Group; Action to Control Cardiovascular Risk in Diabetes Study Group; ADVANCE Collaborative Group; Duckworth et al., 2009; Zoungas et al., 2010). Additionally, a review by Fullerton et al. notes studies that have found a benefit in intensive glycemic control in reducing the risk of diabetic microvascular complications have been of younger patients at earlier stages of the disease (2014). These findings highlight the fact that even tight control of glycemia does not guarantee freedom from the complications of diabetes and suggest that research into strategies that block the adverse effects of hyperglycemia are warranted (Ramasamy, 2011). Given the high rate of prediabetes and undiagnosed diabetes, as well as the number of people who are older and have had the disease longer, new approaches that stop or reverse the complications of diabetes are greatly needed.

Treatment can only benefit those who know they have diabetes. Early detection is key to preventing acute and chronic complications. However, hyperglycemia is typically

asymptomatic, and diabetes may not even be diagnosed until complications occur, at which point serious damage to micro- and macrovascular tissue may already have occurred (American Diabetes Association, 2014; International Diabetes Foundation, 2013). It is estimated nearly one-fourth of the total United States population have undiagnosed diabetes (American Diabetes Association, 2014). With this great number of individuals unaware of what damage may be taking place in their bodies, it is imperative that additional treatment options be investigated to treat the complications of diabetes in cases where it was not prevented or diagnosed and treated early. Prevention is the best course of action, but given the growing number of diagnosed and undiagnosed cases of diabetes, it is necessary to elucidate treatments that can help those who are already afflicted with this disease.

II. Protein Glycation in Diabetes

Hypotheses to explain vascular complications of diabetes. It is clear that hyperglycemia causes microvascular and macrovascular complications. There is much research into the exact mechanisms responsible for the damage to vasculature and tissues of those with diabetes. Several hypotheses have been developed and continue to be researched. Four of these hypotheses include increased polyol pathway flux, activation of PKC isoforms, increased flux of the hexosamine pathway and increased advanced glycation end-product (AGE) formation (Brownlee, 2001).

The polyol pathway is the pathway in which glucose is reduced to sorbitol and converted to fructose (Lorenzi, 2007). In non-diabetic patients, the use of this pathway to metabolize glucose is small. However, during hyperglycemia, increased levels of glucose can trigger increased enzymatic conversion of glucose to sorbitol, resulting in greater flux

of the pathway; this leads to greater amounts of sorbitol, which does not easily diffuse across cell membranes and can damage microvascular cells (Brownlee, 2001; Lorenzi, 2007). Increased flux through this pathway is thought to lead to increased formation of methylglyoxal (a precursor to AGE) and diacylglycerol (DAG) (Brownlee, 2001). It is also hypothesized increased amounts of sorbitol can lead to the overexpression of aldose reductase, the rate-limiting enzyme in the polyol pathway (Lorenzi, 2007; Brownlee, 2001). Studies that have inhibited the polyol pathway in order to prevent diabetic complications have shown inconsistent results; a study in dogs showed aldose reductase inhibition prevented diabetic neuropathy but did not prevent retinopathy or basement membrane thickening in the retina, kidney or muscle (Engerman, Kern & Larson, 1994). It may be that this pathway is more important in some species than others, and some have questioned its relevance in humans in regards to the development of diabetic complications (Brownlee, 2001).

The activation PKC isoforms by DAG is one of the most studied pathways related to the pathology of hyperglycemia (Geraldes, 2010). PKC is important in many cellular functions and signal transduction pathways (Geraldes, 2010). In hyperglycemia, DAG synthesis is increased during glycolysis; DAG can also be indirectly elevated through increased activity of the polyol pathway and through ligation of AGE receptors (Brownlee, 2001). DAG increases activation of several PKC isoforms, which can lead to retinal and renal blood flow abnormalities and may also decrease production of nitric oxide in the kidneys (Geraldes, 2010, Brownlee, 2001). In addition, PKC isoforms altered by diabetes cause endothelial dysfunction, vascular permeability, changes in vessel dilation and basement membrane thickening (Geraldes, 2010). Thus the DAG-PKC

pathway is involved in the development of both the microvascular and macrovascular pathologies found in diabetes.

In a state of hyperglycemia, excess glucose may be shunted into the hexosamine pathway (HSP). The HSP normally has a small role in the metabolism of glucose, but during hyperglycemia, its use is increased, which is hypothesized to contribute to the development of insulin resistance and vascular complications associated with diabetes (Brownlee, 2001; Buse, 2006). It is also thought that hyperglycemia-mediated increase in flux through the HSP facilitates changes in gene expression and protein function, although the exact mechanisms are unclear (Brownlee, 2001).

Protein glycation and formation of AGEs. Another important area of research into the mechanisms responsible for the microvascular and macrovascular complications of diabetes is protein glycation and AGE formation. Among other targets, proteins in blood plasma are susceptible to glycation. The glycation reaction was first described by a French biochemist named Louis Maillard, who observed a browning reaction when heating glycine with glucose (Maillard, 1912; Simm, 2015). When plasma glucose concentrations are high, sugars can bind non-enzymatically to plasma proteins, such as albumin and hemoglobin. It should be noted that glycation differs from glycosylation, which is an enzymatic reaction (Gkogkolou & Bohm, 2012).

Glycation of proteins can lead to the formation of AGEs. Non-enzymatic glycation occurs when the aldehyde or ketone group of a reducing sugar binds to a free amino acid group of a protein, commonly a lysine residue, which forms a Schiff's base (Basta, 2004). The Schiff's base reaction is reversible but may undergo further rearrangements to form a ketoamine called an Amadori product (Basta, 2004; Simm,

2015). The resulting Amadori product is still reversible, but is more stable, than the Schiff's base. Amadori products can form highly reactive carbonyl compounds such as 3-deoxy-glucosone, glyoxal and methylglyoxal, which react with free amino acid groups to form intermediate glycation products (Basta, 2004). These intermediate products then undergo a slow, complex, irreversible series of reactions to form AGEs (Basta, 2004; Simm, 2015). AGEs accumulate in tissues and because of their stability, levels do not decrease even after hyperglycemia is corrected (Makita et al., 1991).

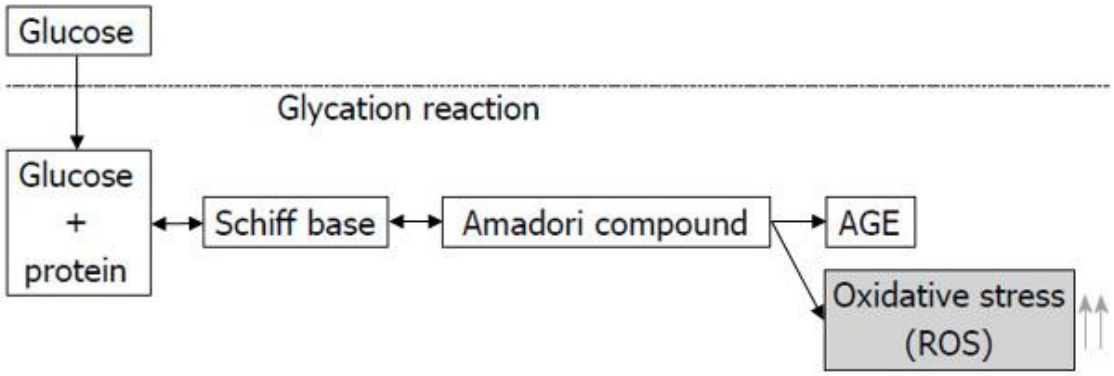

Fig. 1 The formation of advanced glycation end products (AGEs). Adapted from Kawahito, Kitahata & Oshita, 2009

Non-diabetic patients have very low levels of protein glycation, however, diabetic patients have much higher rates of glycation (Austin et al., 1987) as AGEs form more rapidly during chronic hyperglycemia (Ramasamy, 2011). The rates of glycation differ between plasma proteins; this difference is thought to be due to the length of biological half-lives and susceptibility of each protein to glycation (Austin et al., 1987). Proteins such as albumin and immunoglobulins, which have the longest half-lives, show the greatest rate of glycation (Austin et al., 1987).

Albumin is the most abundant protein in human plasma and has several important physiological functions. Made in the liver, 50% - 60% of total plasma protein is comprised of albumin (Kim & Lee, 2012). This protein plays a role in fluid balance between body compartments, transport of hormones, fatty acids, and metabolites in the blood, regulation of the oncotic pressure of plasma, and is also known to behave as an antioxidant (Kim & Lee, 2012; Rondeau & Bourdon, 2011). In regards to its antioxidant capacity, albumin is responsible for the majority of free radical trapping and may protect other proteins from glycation during the initial onset of diabetes by limiting oxidative damage (Kim & Lee, 2012; Rondeau & Bourdon, 2011). Albumin is susceptible to glycation due to its long half-life and sheer abundance in the plasma; in healthy individuals glycated albumin is between 1% - 10% (Shaklai, Garlick & Bunn, 1984), while in diabetic individuals it is increased two to three times this amount (Rondeau & Bourdon, 2011) with some levels reaching greater than 90% in diabetics with poor glucose control (Kisugi et al., 2007).

Glycation modifies the tertiary structure of albumin and increases its molecular weight which impairs its function (Shaklai, Garlick & Bunn, 1984). Albumin is primarily glycated at the lysine-525 residue with several other lysine residues also susceptible to glycation (Garlick & Mazer, 1983). Glycated albumin is directly associated with ROS production, which spurs oxidation and accelerates the production of AGEs (Kim & Lee, 2012). Some *in vivo* studies have shown inhibition of albumin glycation can help prevent the progression of diabetic complications (Rondeau & Bourdon, 2011).

Another plasma protein susceptible to glycation is hemoglobin. This component of erythrocytes was the first glycated protein to be discovered and its measurement is

widely used as a tool to monitor glycemic control (Gkogkolou & Bohm, 2012; Kim & Lee, 2012). There are several different fractions that make up the group of hemoglobins which together are known as HbA_1, with the most glycated hemoglobin being HbA_{1c} (Kilpatrick, 2000). Hemoglobin is glycated at the N-terminal valine and internal lysine amino groups; due to the relative stability of this glycation reaction and the average lifespan of erythrocytes (120 days), the measurement of HbA_{1c} can indicate the level of glycemic control over the last 8-12 weeks (Simm, 2015; Soranzo, 2011).

Both glycated albumin and glycated hemoglobin have been described in detail due to their utility in monitoring and assessing plasma glucose control in individuals with diabetes. While using HbA_{1c} to monitor glycemic status and control has been the most widely used method, some have suggested the measurement of glycated albumin may also be useful, especially in individuals with anemia, impaired renal function and gestational diabetes (Kim & Lee, 2012; Rondeau & Bourdon, 2011). Due to the shorter half-life of albumin, glycated albumin measurements could provide useful information about glucose control and treatment effectiveness over a more recent time period, about 2-4 weeks (Rondeau & Bourdon, 2011).

It should be noted while there is research into these four different hypotheses of mechanisms that lead to microvascular and macrovascular damage due to hyperglycemia, they are not separate entities. Each is related; increased flux of the polyol pathway can lead to production of methylglyoxal, an AGE precursor, and DAG, which activates PKC (Brownlee, 2001). Ligation of AGE receptors can also indirectly activate PKC isoforms (Brownlee, 2001). Some have speculated there is a common element linking these four mechanisms; all processes are induced by an overproduction of superoxide by the

mitochondrial electron-transport chain caused by hyperglycemia (Brownlee, 2001). If this is the case, it is suggested that interrupting the overproduction of superoxide could possibly normalize the mechanisms discussed (Brownlee, 2001).

Pathological effects of AGEs. AGEs are thought to contribute to many major diseases and disorders including insulin resistance, diabetes and its complications, kidney disease, cardiovascular disease, Alzheimer's disease, Parkinson's disease, cataracts, autoimmune diseases and endocrine disorders (Korwar et al., 2015; Manigrasso et al., 2014; Ottum & Mistry, 2015; Simm et al., 2015). Formation of AGEs is a normal part of metabolism, and levels increase with age. However, defense systems have evolved that maintain AGE homeostasis in healthy animals, including endogenous and exogenous antioxidant systems, enzymatic degradation, clearance in the kidneys and receptor-mediated cell uptake and degradation (Ottum & Mistry, 2015). During a state of hyperglycemia, these defense systems are overrun and the process of glycation and formation of AGEs are accelerated (Kellow et al., 2014). In addition to hyperglycemia, formation of AGEs is promoted by oxidative stress, sedentary lifestyles, smoking and diets high in fat, AGEs, and excess calories (Alam, Ahmed & Naseem, 2015; Kellow et al., 2014; Korwar, 2015; Ottum & Mistry, 2015). Once AGEs are formed and begin to accumulate in the body, they exert pathological effects which are thought to contribute to the complications associated with diabetes, as well as insulin resistance thereby leading to the pathogenesis of type 2 diabetes (Korwar, 2015; Ramasamy 2011). In diabetic rats, AGEs accumulate at sites where known diabetic vascular damage occurs - retinas, renal glomeruli and peripheral nerves (Berlanga et al., 2005). Diabetics have substantially higher levels of AGEs in the plasma as compared to nondiabetic individuals (Korwar,

2015). AGEs build up in extracellular spaces and within vessel cell walls and they have a distinctive yellow-brown color and produce reactive oxygen species (ROS) (Brownlee, Cerami & Vlassara, 1988). ROS created by AGEs promote production of more AGEs thus beginning a destructive cycle (Farmer & Kennedy, 2009). AGEs are composed of a large number of different chemical structures with N-q-carboxy-methyl-lysine (CML) as the predominant type of AGE that accumulates in humans (Reddy et al., 1995).

There are several ways in which AGEs cause pathological damage. One important pathway in the pathogenesis of diabetes is the receptor-mediated AGE-RAGE interaction. RAGE is a molecule belonging to the immunoglobulin superfamily that has multiple ligands, including AGEs, and is implicated in causing oxidative stress and activating pro-inflammatory pathways (Basta, 2004; Ramasamy, 2011). Thus, it is via RAGE that AGEs can activate signal transduction cascades and mechanisms that cause cell stress, dysfunction, damage and changes in gene expression to specific organ tissues (Ramasamy, 2011). Under normal conditions, RAGE appears to be important in AGE degradation and as a defense against infection, injury or inflammation (Basta, 2004; Ramasamy, 2011). However, when AGEs, as well as other pro-inflammatory ligands, accumulate in the body, they promote increased RAGE expression, which in turn increases oxidative stress and the activation of the signal transduction cascades mentioned, thus promoting the development of chronic disease (Ramasamy, 2011).

Atherosclerosis is a major vascular complication of diabetes. The vascular endothelium is important in regulating vessel permeability, blood flow and vessel tone (Basta, 2004). During chronic hyperglycemia, and the resulting formation of AGEs, the endothelium is exposed to AGEs, which bind to RAGE, and cause increased permeability

and transit of macromolecules through the endothelium (Esposito et al., 1989). The AGE-RAGE interaction also depletes antioxidants, such as glutathione and Vitamin C (Bierhaus et al., 1997), which in turn further promotes the generation of ROS (Yan et al., 1994). AGEs also interfere with the bioavailability of the important vasodilator nitric oxide (Bucala, Tracey & Cerami, 1991) and promote the expression of powerful vasoconstrictors, thus disrupting endothelial control of vascular tone (Quehenberger, 2000). Experimental evidence also suggests AGEs cause the release of inflammatory cytokines and growth factors and promote adhesion molecules on vascular cells (Basta, 2004). The combined effect of increased permeability, loss of vascular tone control, release of pro-inflammatory molecules, and an increase in adhesion molecules in vascular cells triggers chronic vascular inflammation and an ongoing cycle of cell damage and dysfunction, aided by the release of inflammatory peptides and other ligands of RAGE (Basta, 2004). Chang et al. found that levels of AGEs are positively correlated with lipid profiles and atherosclerotic characteristics; in fact, their research found subjects with higher AGEs levels had higher total cholesterol, triglycerides, LDL-C and lower HDL-C and that levels of circulating AGEs may be a better indicator of atherosclerotic lesions than either fasting plasma glucose or HgA_{1c} (2011).

As mentioned, AGE-mediated RAGE activation has also been implicated in the microvascular complications of diabetes. The same processes described for vascular damage occur at the microvascular level. In the development of retinopathy, concentrations of RAGE in the retina are increased (Barile, 2005). RAGE is thought to regulate several pathophysiological responses by Müller glia cells in the retina in response to stress caused by diabetic conditions (Zong, 2010). Müller glia cells are

activated by RAGE and promote the downstream production of cytokines, which contribute to inflammation (Zong, 2010).

AGEs have been implicated in the pathogenesis of diabetic nephropathy (Bolton et al., 2004) and neuropathy (Bierhaus, 2004). Both AGEs and RAGE are found in higher amounts in the kidneys of diabetics as compared to non-diabetics, with a greater accumulation in the glomerulus, both in the podocytes and the endothelial cells (Tanji et al., 2000). In diabetics, a high level of AGEs, especially CML, have been found in the peripheral nerves (Bierhaus, 2004). Moreover, Bierhaus et al. found greater RAGE expression in cells near AGE accumulation in peripheral nerves of diabetics with neuropathy (2004).

Therapies to prevent, reduce or degrade AGEs. Several possible strategies have emerged and are being researched to prevent, reduce or break down AGEs. These strategies include drugs that prevent AGE formation such aminoguanidine and pyridoxamine. Aminoguanidine showed promise in animal studies by trapping early glycation products, however, human trials were halted after adverse effects were experienced by subjects (Bolton et al., 2004). Some research suggests calorie restriction may reduce levels of AGEs, most likely by reducing the amount of dietary AGEs (dAGEs) consumed (Vlassara & Striker, 2013). Lastly, nutraceuticals, vitamins, minerals and other nutrients, are being investigated for their antioxidant properties; these compounds may help limit AGE formation by decreasing oxidation that spurs further AGE formation and oxidative damage (Gkogkolou & Bohm, 2012). Some of these antioxidants include ascorbic acid (vitamin C), α-tocopherol, niacinamide and zinc, which have been shown to prevent glycation of albumin *in vitro* (Gkogkolou & Bohm,

2012, Tupe et al., 2015). Vitamin C supplementation greatly reduced protein glycation in non-diabetic individuals (Davie, Gould & Yudkin, 1992; Vinson & Howard, 1996). Given that AGEs are an important pathogenic factor in the development of diabetes, continued research is needed to discover ways to reduce or break down AGEs in the body. As highlighted earlier, many individuals are unaware they have diabetes and thus incur damage caused by hyperglycemia and accumulation of AGEs. Prevention of diabetes is the best course of action, however, when diabetes has developed and treatment is needed, strategies to reduce the microvascular and macrovascular damage that leads to diabetic complications is of utmost importance.

III. Birds as Models of Diabetes

Animal models of diabetes. To fully understand the role of hyperglycemia and protein glycation in the pathogenesis of the complications of diabetes, appropriate animal models are needed. Due to the slow progression of the disease, in which it can take years for symptoms of complications to manifest, conventional animal models, such as short-lived mice and rats, do not provide data that is comparable to humans (Szwergold & Miller, 2013). Even longer-lived animals, such as monkeys and pigs, can pose logistical research problems, making long-term study both difficult and expensive (Szwergold & Miller, 2013). In either case, hyperglycemia has to be induced or inbred, which results in sick animals that are difficult to maintain and care for (Szwergold & Miller, 2013). In light of these difficulties, another model has been suggested that eliminates many of these problems - birds.

While in the past birds have been used to study the process of aging, because of certain biochemical characteristics, it has been proposed birds would also provide a

useful model of diabetes (Szwergold & Miller, 2013). Birds naturally have high plasma glucose levels (Beuchat & Chong, 1998; Braun & Sweazea, 2008) and generally have low sensitivity to insulin-mediated glucose uptake (Shiraishi et al., 2011; Sweazea & Braun, 2005; Sweazea, McMurtry & Braun, 2006), however, birds do not suffer the same pathologies of hyperglycemia and insulin resistance that mammals do as the high glucose concentrations are a normal aspect of their physiology. Thus it is apparent birds may possess defense mechanisms that protect them from their naturally high circulating glucose and this is evidenced by the fact that birds outlive mammals of comparable body size by up to three times (Braun & Sweazea, 2008; Holmes, et al., 2001). Birds are healthy and pathology-free, despite sustained naturally high plasma glucose levels, and show that it is possible to live with high glucose concentrations and not develop complications. Discovering what protects birds from the deleterious consequences of high plasma glucose concentrations would aid in creating new approaches to preventing and treating diabetes and its related complications in humans.

Variations in glucose homeostasis between birds and mammals. Understanding the differences in physiology and biochemical mechanistic function between birds and mammals is important in determining what mechanisms protect birds from the effects of maintaining high plasma glucose concentrations. Carbohydrate metabolism is of interest when studying birds as models of diabetes. Some research has shown that birds preferentially use fatty acids as an energy source for flight (Jenni & Jenni-Eiermann, 1998), however, despite this, they maintain high levels of plasma glucose compared to mammals of similar body size (see review: Braun & Sweazea, 2008; figure 2). Healthy birds maintain plasma glucose concentrations of 150 mg/dL or more, with some birds,

such as hummingbirds, maintaining levels as high as 800 mg/dL (Beuchat & Chong, 1998; Harr, 2002).

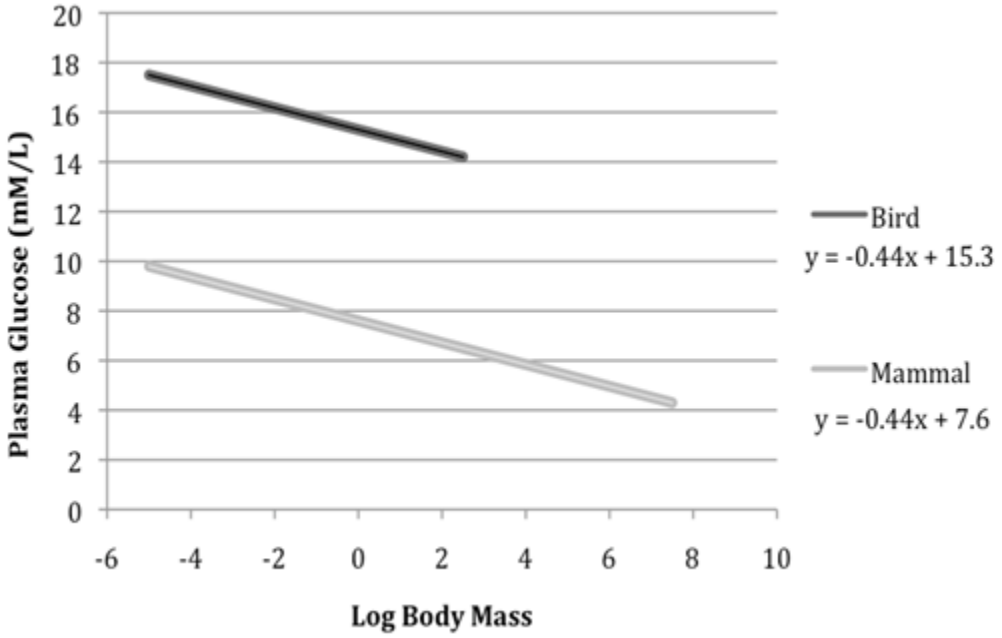

Fig. 2 The relationship between plasma glucose concentrations and log body mass (LBM in kg) for birds (n=97) and mammals (n=162). Data were extracted from the International Species Information System (ISIS), Physiological Data Reference Values 2002. Adapted from Braun & Sweazea, 2008 (J. Zuck thesis, 2015)

The same carbohydrate metabolic pathways that operate in mammals also appear to operate in birds (Pollock, 2002). Glucose is derived from the metabolism of ingested carbohydrates and is used by birds for many purposes, mainly for the production of energy through oxidation, glycogen and fatty acid synthesis, the synthesis of other important metabolites, such as amino acids and vitamin C, and to fuel take-off and short flights (Braun & Sweazea, 2008). Glucose is absorbed either by carrier-mediated transport into and out of enterocytes and/or by passive diffusion through intercellular spaces between enterocytes (Braun & Sweazea, 2008; Pollock, 2002). For many avian species, passive diffusion may be the more important pathway for glucose absorption

since the cost of energy is low. In fact, birds such as nectar-eating lorikeets, yellow-rumped warblers and house sparrows predominantly absorb glucose by passive diffusion (Afik, McWilliams & Karasov, 1997; Karasov & Cork, 1994). Carrier-mediated absorption, in contrast, uses adenosine triphosphate (ATP) to power transport proteins (Braun & Sweazea, 2008). Regardless of absorption pathway, once it is absorbed, glucose can be metabolized through glycolysis and lead to the synthesis of ATP; one molecule of glucose yields 38 molecules of ATP (Pollock, 2002). Also, approximately one-third of the glucose absorbed is converted to lactate, an important precursor for gluconeogenesis (Riesenfeld et al., 1982). Interestingly, glucose absorption of birds matches the amount of glucose in the diet; for example, the nectarivorous Rufous hummingbird has a glucose uptake that is 15 times greater than an omnivorous chicken and 50 times greater than a carnivorous loggerhead shrike (Pollock, 2002).

In mammals, once glucose is absorbed and in circulation, uptake into cells is important in maintaining glucose homeostasis. Glucose is an essential energy source for all cells in animals and it is transported into cells by facilitated diffusion across plasma membranes, a process mediated by glucose transporter proteins (GLUTs) (Suzuki & Kono, 1980). 12 tissue-specific GLUT isoforms (GLUT 1-12) have been identified in mammals (Carayannopoulos et al., 2000; Doege et al., 2001; Gould and Holman, 1993; McVie-Wylie et al., 2001; Phay et al., 2000; Rogers et al., 2002). The isoform GLUT-4 is a major component of insulin-stimulated glucose uptake into skeletal muscle and adipose tissues in mammals (Kern et al., 1990). Much of the research into GLUT transport proteins has come from work with mammal models, therefore less is known about how glucose enters cells in birds (Sweazea & Braun, 2005). Currently, only four GLUT

isoforms similar to human GLUTs have been identified in birds (GLUT 1-3, 8); insulin-specific glucose transport proteins, such as GLUT-4, have not been identified in birds (Seki et al., 2003; Sweazea & Braun, 2006).

Skeletal muscle plays an important role in glucose homeostasis in mammals. GLUT-4 is expressed in this tissue and is estimated to clear 70-75% of postprandial glucose from the blood (Sweazea & Braun, 2005; Tokushima et al., 2005). Due to the high plasma glucose levels observed, it has been questioned whether or not an insulin-responsive transporter like GLUT-4 is present in bird skeletal muscle. Few studies have addressed this question. Seki et al. looked to identify GLUT-4 expression in tissues from broiler chickens; GLUT-4 was not detected and no GLUT-4 homolog could be identified (2003). Sweazea and Braun likewise found no GLUT-4 gene or expression in skeletal and cardiac muscle from English sparrows (2005). Tokushima et al. note that a GLUT-4-like gene sequence has not been found in either chicken expressed sequence tag (EST) or genome databases (2005). In the absence of a GLUT-4-like transporter, some have questioned if an alternative insulin-responsive transporter exists in birds (Seki et al., 2003; Tokushima, 2005). The isoform GLUT-8 has been shown to be insulin-responsive and expressed in various tissues such as the heart, kidney, brain, muscle and liver (Seki et al., 2003). Seki et al. found that GLUT-8 was expressed highly in chicken kidney and moderately in the brain, adrenal, spleen, lung and pancreas, but it was barely detectable in the liver, skeletal muscles, adipose tissue or heart (2003). It is hypothesized the lack of a GLUT-4-like transporter, and to a lesser extent, the low expression of GLUT-8, in part contributes to the hyperglycemia and resistance to insulin-mediated glucose uptake

observed in birds (Seki et al., 2003; Sweazea & Braun, 2005; Sweazea, McMurtry & Braun, 2006).

The most distinct difference in carbohydrate metabolism between mammals and birds is in the hormonal control of glucose (Pollock, 2002). Instead of the hypothesis that GLUT-4 is a contributing factor to hyperglycemia and insulin resistance, it might be more accurate to say that in order to maintain high levels of plasma glucose and insulin insensitivity, GLUT-4 is not expressed in birds (Sweazea & Braun, 2005). High glucose concentrations and insulin resistance are the hallmarks of type 2 diabetes in humans but appear to be normal for birds. Insulin is the dominant pancreatic hormone in mammals, however, this is not the case in birds, although there are interspecies variations in hormonal control (Pollock, 2002). In the fed state, the hormone insulin is responsible for lowering blood glucose levels by increasing the number of glucose transport carriers and by binding to insulin receptors on cells to signal the translocation of glucose transport proteins from intercellular spaces to the cell wall (Shepard & Kahn, 1999; Suzuki & Kono, 1980). Any defect in this pathway can lead to insulin resistance and result in hyperglycemia.

Insulin is an anabolic hormone and drives fuel storage, as either glycogen or fat (Hickey et al., 2011) and suppresses gluconeogenesis, glycogenolysis and lipolysis. Glucose is the trigger for insulin release in mammals, however, it is not the main trigger for insulin release in birds; even when released, birds are generally resistant to the effects of insulin as high concentrations are required to stimulate glucose uptake into cells (Hazelwood, 1973; Sweazea & Braun, 2005; Tokushima et al., 2005). Interestingly, despite having higher concentrations of plasma glucose, birds maintain plasma insulin

levels comparable to mammals (Simon, 1989). The resistance to insulin observed in birds may have to do with the fact that free fatty acids are preferentially used for energy during flight; using fatty acids may inhibit the insulin-signaling pathway and may also down-regulate insulin receptors (Jenni & Jenni-Eiermann, 1998). A study by Kuzmiak et al. showed a preference for fatty acid oxidation even at the level of isolated mitochondria from the pectoralis muscles of house sparrows (2012). While in mammals lipolysis is inhibited by insulin, in birds insulin does not suppress lipolysis (Pollock, 2002). Thus, the need for birds to power and sustain long flights with fatty acids may explain the relative resistance to insulin and an apparent greater sensitivity to glucagon found in birds (Hickey et al., 2011).

Glucagon is the counter-regulatory hormone of insulin. While birds show resistance to insulin and its effects, they show greater sensitivity to glucagon than mammals (Hazelwood, 1973). This hormone is catabolic, and as such, stimulates glycogenolysis, gluconeogenesis and lipolysis to release glucose, triglycerides, glycerol and free fatty acids into the blood to be used as substrates for energy several hours postprandial or in a fasting state (Braun & Sweazea, 2008; Pollock, 2002). Glucagon appears to be the dominant pancreatic hormone that maintains metabolic homeostasis in birds, with as much as 10 to 80 times more glucagon circulating as compared to mammals (Hazelwood, 1989; Pollack, 2002). This physiological difference can be traced back to the islets of Langerhans in the pancreas; in birds, there are a greater number of alpha cells, which secrete glucagon, than there are beta cells, which secrete insulin (Ruffier, Simon & Rideau, 1998). As in mammals, glucose inhibits the release of glucagon in birds (Ruffier, Simon & Rideau, 1998). While in mammals the ratio between

insulin and glucagon seeks to balance anabolic and catabolic phases, in birds this ratio tends to favor catabolism for an ever ready supply of energy to fuel a high metabolism; the normal insulin/glucagon ratio is 1 to 2 or lower (Karasov & Cork, 1994; Karasov & Levey, 1990).

When more glucose is consumed than is immediately needed, excess glucose is converted to and stored as glycogen in the liver and muscles. Glucagon triggers the conversion of glycogen back into glucose when needed (Pollock, 2002). Liver glycogen is utilized during short-term fasts to provide glucose to the brain and other organs that preferentially utilize glucose (Pollock, 2002). In birds, glycogen stored in muscle remains relatively stable during short-term fasts, however, glycogen levels decrease greatly during prolonged fasts, especially in carnivorous birds (Pollock, 2002). On the whole, birds store less glycogen in muscle than do mammals, which given the high levels of plasma glucose in birds means glucose stays in the blood instead of being converted to glycogen (Braun & Sweazea, 2008; Hickey et al., 2011). Glycogen release appears to be activity-dependent, depending on the degree of activity and the species, glycogen stores can be used up within 15-30 minutes to a few hours (Pollock, 2002). However, in birds, glycogen is of little importance during long bouts of flying, which is fueled by fatty acids (Jenni & Jenni-Eiermann, 1998). A unique feature among birds as compared to mammals and other vertebrates is the presence of a glycogen body in the lumbosacral region (Moller & Kummer, 2003). It is thought this tissue forms glycogen taken up by GLUT-1, however, the stimulus of release of glycogen from the glycogen body has not yet been determined and the function and purpose of this tissue is still not understood (Braun & Sweazea, 2008).

In most birds, gluconeogenesis begins several hours after eating due to low glycogen stores coupled with the demands of high metabolic rates (Braun & Sweazea, 2008; Pollock, 2002). The process occurs mostly in the liver and somewhat in the kidney; the primary substrates for gluconeogenesis are glycerol and lactate (Hazelwood & Lorenz, 1959; Klasing 1998; Pollock 2002). In birds, gluconeogenesis works to maintain plasma glucose concentrations at a constant level despite feeding frequency or dietary intake; more than 70-75% of glucose in circulation is produced by gluconeogenesis and released from the liver (Braun & Sweazea, 2008; Pollock 2002). In fact, after several days of fasting, plasma glucose concentrations will rise to normal levels due to gluconeogenesis (Pollock, 2002). A remarkable example of this is found in male emperor penguins; after a 115 day breeding fast, no significant alterations in plasma glucose levels are observed (Groscolas, 1986).

Several avian organs have notable differences compared to mammals in regards to glucose homeostasis. As just described, the liver plays a major role in maintaining the high levels of plasma glucose concentrations observed in birds at a constant level. The liver accomplishes this task by being the site of glycogenolysis and gluconeogenesis, which contribute a majority of the circulating glucose (Braun & Sweazea, 2008). Notably, some bird species have lower numbers of insulin receptors in the liver (Rideau, 1998), which invariably contributes to insulin resistance and further contributes to their naturally high plasma glucose levels.

In mammals, the pancreas performs an important role in glucose homeostasis. Pancreatic activity in birds differs somewhat from its mammalian counterpart. As noted, in birds the pancreas has fewer beta cells and more alpha cells, which means more

glucagon is produced and secreted than insulin (Ruffier, Simon & Rideau, 1998). Granivorous birds, such as chickens, have about 1/6 the amount of insulin in the pancreas as compared to mammals, while glucagon levels are two to three times higher (Harr, 2002). Glucose is required in large doses to stimulate insulin release and in fact, when the beta cells of the avian pancreas are chemically destroyed, no significant effect on plasma glucose is observed (Stellenwerf & Hazelwood, 1979). A similar action in mammals leads to hyperglycemia and ultimately diabetes. Of interest, removing the pancreas from some bird species does not result in the development of diabetes (Hazelwood, 1989). In fact, after pancreatectomy, transient hyperglycemia may result, but plasma glucose levels return to normal after a short amount of time (one week in white domestic ducks) (Koppanyi et al., 1926). The ability of birds to maintain plasma glucose levels after pancreatectomy suggests that an extra-pancreatic source of both insulin and glucagon may exist (Colca & Hazelwood, 1976).

The kidneys are another organ system that functions to maintain glucose homeostasis in birds. What is interesting about the kidneys is that although birds have very high plasma glucose, no glucose is lost in the urine (Morgan & Braun, 2001). Avian nephrons effectively filter and reabsorb glucose before excretion so that no glucose appears in the urine (Lotz & Martinez del Rio, 2004), in contrast to mammalian kidneys which have a glucose absorption threshold (Gorden, 1997). This suggests that all glucose consumed and absorbed by the intestines and also made through gluconeogenesis is utilized by birds (Braun & Sweazea, 2008).

What might be the physiological advantage for birds to have high plasma glucose levels and insulin resistance, as has been discussed? Several benefits have been

hypothesized. As noted, birds store less glucose as glycogen and rather use the blood for a kind of reserve of readily available glucose, which may help supply the demands of high metabolic rates (Hickey et al., 2011). Also, glycogen, when stored in muscle, is hygroscopic, meaning it attracts and stores two and a half to three times its weight in water which makes glycogen heavy and not the best fuel storage method for birds that engage in flight and migration, where excess weight is not ideal (Hickey et al., 2011; Kuzmiak et al., 2012). Less glycogen stored in muscle means there is more room for mitochondria to help power flight and less weight to carry (Hickey et al., 2011). The insulin to glucagon ratio observed in birds tends to favor gluconeogenesis and lipolysis which makes fuel constantly available to not only sustain the higher rate of metabolism observed in birds but to supply adequate fuel for flight and other metabolically stressful activities such as egg laying and fasting during molting (Pollock, 2002). In addition, insulin resistance may protect tissues from oxidative damage that may occur when too much glucose is taken up by cells during hyperglycemia (Sweazea & Braun, 2005).

Protein glycation and AGEs in birds. In mammals, glycation of proteins in the presence of chronic hyperglycemia and subsequent formation of AGEs leads to the development of microvascular and macrovascular pathologies associated with diabetes. Given that birds have higher metabolic rates, body temperatures and levels of plasma glucose in general as compared to mammals, it would be expected that birds also have higher levels of protein glycation and AGEs. However, available evidence suggests birds generally have lower rates of protein glycation and AGE formation despite these conditions (Holmes, Fluckiger, & Austad, 2001; Iqbal et al., 1999; Kita, 2011; Machin et al., 2003). There is little research into protein glycation in birds, however, some specific

glycation products have been studied; pentosidine, glycated hemoglobin and glycated albumin.

Pentosidine is an AGE that is formed by glycation of collagen, a structural protein, and has been used as a biomarker for aging since it increases in tissues with advancing age (Iqbal et al. 1999; Vleck, Haussmann & Vleck, 2007). Iqbal et al. found despite high levels of plasma glucose, pentosidine content in the skin of chickens was markedly lower than what is observed in mammals (Iqbal, 1999). It is not clear why this is the case. One hypothesis is the collagen structure differs between mammals and birds and thus may inhibit non-enzymatic glucose binding or pentosidine formation may be prevented by high levels of uric acid, a powerful antioxidant, that inhibits oxidative steps in the AGE formation process (Klandorf & Iqbal, 1999). Gu et al. also found chickens had 2.3 times lower pentosidine concentrations than humans, which suggests that high plasma glucose levels do not lead to greater AGE formation in chickens as is observed in diabetic humans (2012).

Among birds, hummingbirds have the highest plasma glucose levels (>650 mg/dL), yet have remarkably low levels of glycated hemoglobin, in the range of 2-5% (Beuchat & Chong, 1998). While this range is high among birds, it is not problematic in mammals. Duck, chicken and turkey glycated hemoglobin levels have been measured in the range of 0.5-1.0% (Rendell et al., 1985). One explanation for this occurrence involves structural differences in erythrocytes between mammals and birds. Mammalian erythrocytes lack a nucleus and are fairly permeable to glucose, while bird erythrocytes are nucleated and are less glucose permeable (Beuchat & Chong, 1998; Holmes, Fluckiger, & Austad, 2001). Another factor may be erythrocyte lifespan; bird

erythrocytes have half-lives of 50-70% of mammalian erythrocytes (Beuchat & Chong, 1998; Holmes, Fluckiger, & Austad, 2001). Lower glucose permeability and shorter lifespans of avian erythrocytes mean glycated hemoglobin is more quickly replaced (Hickey et al., 2011).

Interestingly, some research shows the glycation of albumin is comparable between chickens and mammals (Rendell et al., 1985), however, despite this, birds in general exhibit lower overall levels of AGEs than mammals (Holmes, Fluckiger, & Austad, 2001). Rendell et al. measured levels of glycated hemoglobin and albumin in several species of mammals and birds and found chickens had lower levels of glycated hemoglobin (0.54%) than humans (5.76%) yet had higher levels of albumin glycation (2.35%) compared to humans (1.5%) (1985). In a prior study, Anthony et al. found that glycation of avian and bovine albumin appears to be glucose dependent, since rates of glycation increased as glucose concentration increased, which supports earlier evidence of albumin glycation in birds. In humans, albumin is highly glycated and makes up a large part of serum AGEs (Korwar, 2015). Human serum albumin is glycated at surface-exposed lysine residues (Korwar, 2015). In chickens, the predicted conformational structure of albumin differs from mammals with two of the lysine residues available for glycation folded inwards and thus not exposed, making nonenzymatic binding more difficult (unpublished observations).

In mammals, glycated proteins undergo further chemical reactions to become AGEs. AGEs accumulate in tissues and levels do not decrease even after hyperglycemia is corrected (Makita et al., 1991). Kita examined tissue distribution of AGEs in birds by administering radioactive AGEs to chickens and found accumulation was greatest in the

liver, kidney and spleen of the birds (2011). This is interesting given that one of the main functions of these organs is to clear toxins and metabolic waste from the body; perhaps AGEs in chickens are cleared by the liver, kidney and spleen, thus leading to the lower levels of AGEs observed (Kita, 2011). Interestingly, AGEs do not accumulate in the eyes of chickens suggesting other pathways, such as the polyol pathway, may be more important in the pathology of diabetic retinopathy (Kita, 2011). This may in part be due to the structural differences of the eye between mammals and birds, namely, birds have avascular retinas, while mammalian retinas are vascularized (Chase, 1982; Wolberg et al., 1999). Thus, mammalian intra-retinal vasculature is more prone to damage from hyperglycemia than avian avascular retinas.

Overall, the low rates of protein glycation observed suggest birds have evolved and possess adaptations and mechanisms that prevent and/or protect against protein glycation and the formation of AGEs (Holmes, Fluckiger, & Austad, 2001; Szwergold & Miller, 2014). One such adaptation may involve RAGE. There is little research into RAGE in avian species, however, preliminary evidence suggests that RAGE may not be present in birds. Researchers found mourning dove tissues lacked RAGE proteins (F. Eythrib thesis, 2013). A comparison of DNA sequence data between mammals, rodents and birds (chickens, turkeys and zebra finches) found that birds do not possess a homolog to mammalian RAGE (Szwergold & Miller, 2014). Shortly after this study was published, a second appeared showing that only mammals express RAGE (Sessa et al., 2014). Lack of RAGE helps explain how birds protect against protein glycation and the pathological effects of AGEs.

ROS and oxidative stress. Considerable research has focused on oxidative stress and its causes. Oxidative stress results from the formation of highly reactive molecules, ROS, namely the superoxide radical, hydroxyl radical and hydrogen peroxide, which in excess quantities can damage cells and tissues and lead to the loss of physiological function (Vleck, Haussmann & Vleck, 2007). While a chronic excess of ROS can cause damage to tissues and is implicated in many disease states, it is important to note ROS production is a normal part of cellular metabolism and ROS do serve important physiological purposes in maintaining homeostasis in healthy individuals (Finkel & Holbrook, 2000). ROS are important in muscle contraction (Ristow et al., 2009), signaling pathways in the vasculature and other pathways (Brownlee, 2001) and cellular apoptosis (Finkel & Holbrook, 2000). ROS also have an immune system function and help to defend against toxins and invading microorganisms (Finkel & Holbrook, 2000). Intermittent high ROS production brought on by exercise has been shown to be beneficial (Ristow et al., 2009). Mitochondria produce the majority of ROS in animal cells; some limited research suggests animals with longer lifespans have lower rates of ROS production, suggesting longer-lived animals have defense systems to protect against oxidative damage (Barja & Herrero, 2000; Sohal et al., 1989). In fact, in mammals, maximum lifespan is inversely related with metabolic rate and rate of ROS production (Sohal & Weindruch, 1996).

During chronic hyperglycemia in mammals, ROS production (specifically the superoxide radical and hydrogen peroxide) and subsequent tissue damage are increased (Brownlee, 2001; King & Loeken, 2004). This happens in part because chronically elevated levels of plasma glucose cause uncoupling in the electron transport chain which

leads to ROS production (Brownlee, 2001). In addition, elevated glucose levels lead to the production of AGEs which in turn cause oxidative damage (Yao & Brownlee, 2010). The formation of AGEs involves oxidative steps and the interaction of AGEs with their receptors, such as RAGE, cause oxidative stress and damage (Yan, Levine & Sohal, 1997). Both ROS and AGEs cause oxidative damage to tissues and vasculature in mammals (King & Loeken, 2004). Hyperglycemia may also down-regulate antioxidant systems which protect against ROS (West, 2000).

Birds have higher metabolic rates and levels of plasma glucose than mammals, which means they should also have higher levels of ROS production and oxidative stress, however, this does not seem to be the case. Despite high metabolic rates and high plasma glucose, birds live up to three times longer than mammals of comparable body mass (Speakman, 2005). As oxidative stress has been implicated in the process of aging (Vleck, Haussmann & Vleck, 2007) and the production of AGEs, this suggests bird physiology somehow prevents oxidative damage from occurring (Braun & Sweazea, 2008). Some studies in pigeons have shown resistance to ROS formation in the brain, heart and kidney tissues despite high plasma glucose levels (Ku & Sohal, 1993).

Several processes may be at work to prevent oxidative damage in birds. Some evidence shows there may be differences in ROS production between birds and mammals in that birds produce lower levels of ROS (Braun & Sweazea, 2008; Iqbal et al., 1999; Holmes, Fluckiger, & Austad, 2001; Vleck, Haussmann & Vleck, 2007). Birds also appear to have more robust antioxidant systems with higher levels of antioxidants that scavenge ROS, such as superoxide dismutase, catalase, glutathione peroxidase and uric

acid, which help to make cells more resistant to oxidative stress (Braun & Sweazea, 2008; Holmes, Fluckiger, & Austad, 2001; Smith et al., 2011).

The antioxidant uric acid has been positively correlated with a long lifespan and is found at comparatively high levels in humans (Ames et al., 1981). Uric acid plays an important role in the function of vitamin C, a free radical scavenger, as well as being a potent free radical scavenger itself (Ames et al., 1981). Levels of uric acid in humans that are too high, however, can promote oxidative stress and can lead to gout and damage to blood vessels (So & Thorens, 2010). Birds also have high levels of uric acid when compared to mammals of similar body mass (Smith et al., 2011). Uric acid is the main end product of nitrogen metabolism in birds (Harr, 2002; Machin et al., 2004; So & Thorens, 2010), which when inhibited in chickens by a uric acid inhibitor (allopurinol) results in increased oxidative damage (Klandorf et al., 2001). It is produced mainly in the liver and secreted mostly in the proximal convoluted tubules of the kidneys (Harr, 2002). Routine levels of uric acid in bird plasma are in the range of 0.2-0.5 mM (Vleck & Vleck, 2002). Smith et al. found levels of uric acid in mourning doves to be four times higher than in rats (2011). Uric acid concentrations have been proposed to play an important role in the antioxidant systems of birds (Iqbal et al., 1999).

It may also be that birds can limit the consequences of AGEs formed with their antioxidant defenses (Holmes, Fluckiger, & Austad, 2001). Some research suggests greater fitness and total lifetime energy expenditures may help offset the effects of the characteristics mentioned above that cause oxidative damage in mammals (Hickey et al., 2011). Bird dependence on lipid oxidation to fuel flight may also help keep ROS production low since this metabolic pathway results in less ROS production than

carbohydrate metabolism (Kuzmiak et al., 2012). Exogenous antioxidants obtained from the diet, such as carotenoids and various vitamins, may also play a role in the strong antioxidant defense systems of birds, though the true extent of their role needs further examination (Smith et al., 2011).

Humans, under normal conditions, have antioxidants systems in place to prevent accumulation and oxidative damage from ROS (Smith et al., 2011), however, total antioxidant capacity decreases with age (Mecocci et al., 2000). Of note, research conducted with zebra finches found antioxidant capacity did not change over the lifespan (~7 years) of the birds, a characteristic if the same for all birds, could be another reason birds have low rates of oxidative stress and damage (Vleck, Haussmann & Vleck, 2007).

IV. Effect of Diet on Protein Glycation and Formation of AGEs

Dietary factors that promote AGE formation. As discussed, chronic hyperglycemia promotes protein glycation and AGE formation. Consuming a Western-type diet, high calorie, high fat, low fiber, combined with sedentary lifestyle, sets the stage for insulin resistance and chronic hyperglycemia and thus protein glycation and AGE formation. Nutrition also influences levels of ROS, which promote protein glycation; the Western diet increases oxidative stress (Ottum & Mistry, 2015).

An interesting fact about the Western diet is that food processing and high-temperature cooking generate AGEs and these dietary AGEs (dAGEs) are thought to contribute to the overall load of AGEs in the body (Alam, Ahmed & Naseem, 2015; Forbes et al., 2013; Kellow et al., 2014; Ottum & Mistry, 2015; Uribarri & Tuttle, 2006). It is estimated roughly 10% of dAGEs in food are absorbed into circulation and only about 1/3 are excreted by the kidneys, leaving approximately 6% to accumulate in the

body with endogenous AGEs and cause damage (Forbes et al., 2013; Ottum & Mistry, 2015). AGE precursors such as CML and methylglyoxal are also created during food processing and can be absorbed just like AGEs (Alam, Ahmed & Naseem, 2015).

Macronutrient composition. Much interest has been shown in the macronutrient composition of the diet and how it might affect weight, metabolic health and the development of diabetes (Forbes et al., 2013). Each macronutrient – carbohydrate, fat, and protein – has different effects on plasma glucose levels (Kang, Mi & Jun, 2012). Individuals with diabetes need to monitor carbohydrate intake as it directly affects blood glucose levels after meals, but a specific dietary macronutrient composition for diabetics remains debated (Kang, Mi & Jun, 2012). Lower carbohydrate diets have been related to better glycemic control, however, severe restriction of carbohydrates is related to poor glycemic control, most likely because it increases intake of other macronutrients, such as fat (Kang, Mi & Jun, 2012) which in turn promotes gluconeogenesis. High fat diets promote insulin resistance and glucose intolerance in mammals (Bielohuby et al., 2013). Animal models show diets high in fat and simple carbohydrates will induce insulin resistance and oxidative stress compared to a low fat and high complex carbohydrate diet (Barnard et al., 1998; Roberts et al., 2006). Some research indicates high protein diets may be marginally more effective for weight loss (Bueno et al., 2013), but others warn for diabetics, high protein diets, which are typically high in dAGEs, increase the load of AGEs in the body and lead to kidney damage (Uribarri & Tuttle, 2006). While specific macronutrient distribution needs further research, total caloric intake may be more important in the prevention and treatment of diabetes. In a study of Korean adult

diabetics, glycemic control was related to total energy intake and not to macronutrient composition (Kang, Mi & Jun, 2012).

Protective dietary factors. Exogenous dietary antioxidants play a role in the body's innate antioxidant systems to prevent oxidative damage. Diets that include a variety of fruits, vegetables, nuts and seeds are good sources of antioxidants such as vitamins A, C, and E, carotenoids, selenium and zinc (Ottum & Mistry, 2015). Most mammals and birds produce vitamin C endogenously, but humans must consume this powerful antioxidant from food. In addition to its antioxidant role, vitamin C can also bind to glycation sites on proteins and prevent glycation (Davie, Gould & Yudkin, 1992). Supplementation of vitamins C and E in diabetics has shown to decrease protein glycation (Ceriello et al., 1991; Davie, Gould & Yudkin, 1992). Fruits and vegetables contain not only vitamins and minerals but many phytochemicals, including flavonoids, which are thought to be protective as well by preventing oxidative stress and glycation (Stefek, 2011). The flavonoid quercetin, for example, is a potent scavenger of ROS and may prevent cataracts, a common complication of diabetes, by quenching the free radical intermediates involved in the glycation process (Stefek, 2011). Some research suggests quercetin also binds to lysine residues to prevent glycation from occurring (Alam, Ahmed & Naseem, 2015). Zinc, an essential element, has been shown to reduce the formation of Amadori products, thus decreasing the amount of AGEs produced (Tupe et al., 2015). Interestingly, the antioxidants Vitamins A and E, are higher in birds than in mammals (Schweigert et al., 1991). Mourning doves specifically were found to have higher levels of the carotenoid precursor to vitamin A, retinol, and other carotenoids as well as vitamin

E than rats (Smith et al., 2011). Carotenoids are important in birds for plumage and beak color (McGraw et al., 2004).

A diet low in processed and high-heat treated foods may also be protective because it decreases the amount of dAGEs consumed (Forbes et al., 2013; Ottum & Mistry, 2015). Cooking foods at lower temperatures and with moisture helps to decrease the levels of dAGEs, for example, a fried, breaded chicken breast with skin contains about 10,000 kU/100 g AGEs (CML and methylglyoxal) while a skinless, poached chicken breast contains 1,000 kU/100 g (Uribarri et al., 2010). Some evidence shows decreasing dAGEs may be more powerful than calorie restriction for improving health markers such as insulin sensitivity and maintaining glucose homeostasis (Forbes et al., 2013; Ottum & Mistry, 2015). A study by Macías-Cervantes et al. found a low dAGE diet in humans was just as effective at lowering serum AGEs and body fat as exercise (2015).

V. Use of Carnivorous Birds

Current research in avian protein glycation. As discussed, available evidence suggests birds generally have lower rates of protein glycation and AGE formation. There is little research into avian protein glycation. Much of the work that has investigated protein glycation has been done with commercially-bred chickens or other domesticated birds such as ducks and turkeys (Holmes, Fluckiger & Austad, 2002; Szwergold & Miller, 2014). Chickens are easily accessible and are often used as a reference species for comparative studies (Myers & Klasing, 1999). Commercially-bred chickens are granivores, a type of herbivore, meaning they primarily eat seeds and grains and therefore consume a relatively high carbohydrate, low protein, low fat diet (O'Donnell, Garbett & Morzenti, 1978). Other herbivorous birds are frugivores, which prefer fruit, and

nectarivores, that primarily consume nectar. Omnivores consume both plant and animal matter. The jungle fowl from which chickens were domesticated from are omnivores because they eat both plant matter and insects (Myers & Klasing, 1999). Birds such as woodpeckers and cuckoos eat insects primarily and are classified as insectivores, a type of carnivore. Carnivores eat a diet which consists mainly of the flesh and tissues of other animals, which is high in protein and fat (O'Donnell, Garbett & Morzenti, 1978), and is very low in carbohydrate, which is consumed as glycogen or free glucose from prey (Myers & Klasing, 1999). Carnivorous birds, such as bald eagles, obtain meat through predation or scavenging (Newsome, Collins & Sharpe, 2015). For the purposes of research, it is important to examine questions related to glucose regulation by considering variations in avian dietary habits as this may provide further insight into the effects of diet on protein glycation.

Protein glycation in birds of prey. As mentioned, protein glycation has been evaluated in only a few species of birds. These include chickens, ducks, turkeys, hummingbirds and budgerigars (Holmes, Fluckiger & Austad, 2002). At present, native protein glycation has not been evaluated in carnivorous birds of prey. It has been suggested carnivorous animals would be useful models of diabetes since carnivore metabolism induces metabolic reactions similar to pathological abnormalities observed in those with prediabetes or diabetes (Schermerhorn, 2013). Understanding glucose homeostasis and antioxidant capacity in birds of prey are thus necessary to the larger goal of determining how birds protect themselves from high plasma glucose concentrations and protein glycation.

Several key differences exist between birds of prey which consume a high protein, high fat diet and chickens which consume a high carbohydrate, low protein, low fat diet. Birds of prey live longer and age slower than short-lived chickens, which age quickly (Holmes, Fluckiger & Austad, 2002). Birds of prey have higher fasting plasma glucose levels (O'Donnell, Garbett & Morzenti, 1978; Sweazea et al., 2014) and can maintain those levels longer than chickens during a fast or time of stress (Wallner-Pendelton, Rogers & Epple, 1993). Minick found stress and/or fasting has little impact on plasma glucose levels in birds of prey (1986). This is accomplished by continuous gluconeogenesis in both the fed and fasting state; gluconeogenesis maintains stable plasma glucose levels and spares hepatic glycogen, even during a prolonged fast (Migliorini et al., 1973). Continuous gluconeogenesis, which uses amino acids from the diet in the fed state and amino acids from muscle during the fasting state, allows birds of prey to withstand longer periods of time between feeding compared to chickens that must eat more frequently (Pollock, 2002). Interestingly, barn owls given an intravenous glucose challenge maintained high plasma glucose levels for 3.5 hours due to gluconeogenesis compared to chickens, which returned to normal plasma glucose levels within 30 minutes (Myers & Klasing, 1999). Red-tailed hawks also take longer to return to normal levels than chickens after a glucose challenge (Myers & Klasing, 1999). O'Donnell et al. suggest the difference in plasma glucose levels between birds of prey and chickens is due to differences in diet (1978). Some research indicates carnivorous animals adapt poorly to low protein diets due to an inability to down-regulate gluconeogenesis, whereas omnivorous animals adapt more easily to changes in the macronutrient composition of the diet (Myers & Klasing, 1999).

In chickens, glucagon is the predominant pancreatic glucoregulatory hormone, however, some research suggests insulin is more dominant in carnivorous birds (Pollock, 2002; Sweazea et al., 2014). Removal of the pancreas results in hypoglycemia in chickens but in carnivorous birds results in hyperglycemia, glycosuria, and the possible development of diabetes (Harr, 2002; Sweazea et al., 2014; Wallner-Pendleton, Rogers & Epple, 1993). This may be explained by the observance that the pancreatic islets of Langerhans of birds of prey contain more beta cells, which produce insulin (Palmieri & Shivaprasad, 2014; Ruffier, Simon & Rideau, 1998), while the islets in granivorous birds are predominantly alpha cells, which produce glucagon (Pollock, 2002). While insulin concentrations are lower in carnivorous birds than in chickens, insulin in birds of prey is more sensitive to glucose than insulin in chickens, suggesting a higher level of insulin sensitivity in birds of prey (Myers & Klasing, 1999).

Carnivorous birds have higher metabolic rates than omnivores or granivores (Nagy, Girard & Brown, 1999). It might be expected that birds of prey would exhibit higher levels of oxidative stress, due to a higher metabolic rate, than chickens, however, it was found that production of free radicals in red-tailed hawks is comparable to chickens, with both species exhibiting lower levels of free radical production than cows (Conlon, Smith & Gowlett, 1991). Birds of prey have higher reference values for uric acid, which combined with lower free radical production, may explain the lower levels of oxidative stress observed (Harr, 2002).

Human relevance of evaluating protein glycation and antioxidant levels in birds of prey. As discussed, both birds as a class and carnivorous animals, in general, have been suggested to be used as models of diabetes for further research and insight into the

mechanisms of hyperglycemic damage in diabetic humans. Birds of prey are unique in that they fit both criteria – carnivorous birds. Studying birds of prey and how these species avoid diabetes and diabetic complications despite having high levels of plasma glucose while consuming a high protein, high fat diet, similar to the so-called Western diet, could generate ideas for new research into treatment options for the millions of people who are already afflicted with diabetes and the complications caused by protein glycation and AGEs. Specifically, determining what amount of protein glycation in birds of prey is "normal" and what levels of other protective antioxidant factors may be present in circulation, such as carotenoids and vitamin E, is the next logical step.

CHAPTER 3

METHODS

Materials. Serum samples from several birds of prey (Bald Eagle (BE), Red-Tailed Hawk (RTH), Barred Owl (BO), and Great Horned Owl (GHO)) were obtained from The Raptor Center, a rehabilitation center for birds of prey at the University of Minnesota's College of Veterinary Medicine. The high protein, high fat diet of the birds of prey at the center includes rabbits, small birds, mice, rats and chickens. Diet was not supplemented with vitamins or minerals. Blood samples were collected from 24 birds (BE n=6, RTH n=7, BO n=4, and GHO n=7) and centrifuged to separate serum from formed elements. Body temperatures of the birds were also taken using a cloacal probe and body mass was recorded. Samples were shipped from the Raptor Center to Arizona State University overnight on dry ice and stored at -80ºC upon arrival. Chicken serum from one animal was donated from the Department of Animal Care and Technologies at Arizona State University for use as a control. All animal protocols were approved by the Arizona State University and University of Minnesota Institutional Animal Care and Use Committees (Appendices A and B).

Measurement of glucose levels. Plasma glucose levels were measured in all samples using a glucose colorimetric assay kit (Cayman Chemical, catalog #10009582). See Appendix C for protocol. The only deviations from the protocol were that all serum samples were diluted 1:15 with assay buffer prior to the assay to bring the samples within the range of detection and the reactions occurred at 41º C (normal body temperature of birds of prey), rather than mammalian-specific 37º C mentioned in the instructions.

Glucose levels in the chicken serum were not measured as levels for chickens are well-documented in the literature.

Preliminary studies to determine the effects of hyperglycemic conditions on protein glycation. In order to assess the feasibility of incubating serum samples with added glucose to simulate hyperglycemic conditions, two trials were conducted. Previous research in Dr. Sweazea's laboratory showed a high percentage of albumin glycation in samples isolated from a great horned owl after incubating the sample at 41ºC for 24 hours. These findings suggest that either albumin is glycated as a result of oxidation (i.e. exposure to air outside of the body) or that glucose can auto-oxidize the protein. To rule out the possibility for glucose auto-oxidation, one chicken serum sample was incubated for 24 hours at 41ºC in a BioRad Thermocycler and stored at -80ºC until analysis by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) to determine percent glycated albumin. A chicken sample was used for this test as prior research showed very little auto-oxidation of purified chicken serum albumin (J. Zuck Thesis, 2015). Both native glycation and glycation in the incubated serum sample were measured. Similar to the sample from a great horned owl, the preliminary results showed glycation was greater in the chicken serum sample that was incubated for 24 hours at 41ºC as well. Thus a second trial was conducted to see if auto-oxidation could be prevented by adding deferoxamine (DFO), a metal chelator to the serum. For this trial, a sample of great horned owl serum was adjusted to achieve a final total glucose concentration of 400 mg/dL (i.e. hyperglycemic) using a stock solution containing 10 mg/ml glucose prepared in 0.9% saline. A separate aliquot received the same volume of 0.9% saline devoid of glucose. Both solutions were aliquoted in duplicate and 0.4 µl DFO

was added to each. The samples were then incubated at 41°C for 24 hours and resulting protein glycation determined by LC-ESI-MS. Baseline glycation and glycation levels after 24-hour incubation were measured. Results indicated high levels of glycation (>70%) in both incubated samples, suggesting high levels of glucose auto-oxidation even in the presence of the metal chelator. Therefore, experiments designed to expose serum from birds of prey to hyperglycemic conditions are not possible as all samples (saline vs. high glucose added) had very high glycation that is not representative of what happens *in vivo.*

Measurement of native protein glycation. Percent glycation of serum albumin was measured in all serum samples by LC-ESI-MS in the laboratory of Dr. Chad Borges in the BioDesign Institute at Arizona State University. All aliquots extracted were diluted in a 1000:1 ratio (500 µl 0.1% trifluoroacetic acid (TFA) to 0.5 µl serum) and analyzed intact by LC-ESI-MS on a Dionex Ultimate 3000 HPLC equipped with a 1:100 flow splitter connected to a Bruker Maxis 4G quadrupole-time-of-flight (Q-TOF) mass spectrometer. A trap-and-elute form of LC-MS was carried out in which 25 µL diluted samples were loaded at 10 µl/min in 80/20 water/acetonitrile containing 0.1% formic acid (loading solvent) onto a Bruker-Michrom protein captrap configured for bi-directional flow on a 6-port diverter valve. The flow over the captrap was then switched to the micropump, set at 3 µL/min. A multi-step gradient was used: the first step was held at 65% water containing 0.1% formic acid (Solvent A) / 35% acetonitrile (Solvent B) for 30 seconds; the next step was ramped to 45% acetonitrile and held for three minutes; the last step was ramped to 80% acetonitrile and held for 2 minutes. The captrap eluent was directed to the mass spectrometer operating in positive ion, TOF-only mode, acquiring

spectra in the m/z range of 300 to 3000 with a nominal resolving power of ~60,000 m/Δm FWHM. ESI settings for the Agilent G1385A capillary microflow nebulizer ion source were as follows: end plate offset -500 V, capillary -4500 V, nebulizer nitrogen 4 bar, dry gas nitrogen 4.0 L/min at 225 °C. Data were acquired in profile mode at a digitizer sampling rate of 3 GHz. Spectra rate control was by summation at 1 Hz.

Each serum sample eluted over a period of about 0.5 minute; under the above conditions, serum samples ranged in charge state from +33 to +68. Raw mass spectra were averaged across this timeframe, baseline subtracted 0.7, charge deconvoluted with Bruker DataAnalysis 4.1 charge deconvolution software to a mass range of 1000 Da on either side of any identified peak (see Appendix D).

For each species, the peak counts corresponding to the peaks for native albumin (in Da: BE ~66895; RTH ~66753; BO ~67303; GHO ~67351; chicken ~66874), glycated albumin (in Da: BE ~67057; RTH ~66916; BO ~67464; GHO ~67512; chicken ~67036), and doubly glycated albumin (in Da: BE ~67207; RTH ~67074; BO ~67624; GHO ~67674; chicken ~67198) were obtained from the mass spectra and used in the following formula to calculate percent glycated albumin: *Percent Glycated Serum Albumin = {[glycated albumin + 2(doubly glycated albumin)] / [native albumin + glycated albumin + doubly glycated albumin]} * 100*.

Measurement of potential vasoprotective factors. In general, birds reportedly have higher circulating antioxidants as compared to mammals (Smith et al., 2011). These antioxidants may protect birds from protein glycation in vivo. Uric acid levels were measured in all samples (QuantiChrom uric acid assay kit, catalog #DIUA-250). See appendix E for the protocol. The only deviation from protocol is all samples were diluted

1:2 to bring them within the range of detection. Various exogenous antioxidants were also measured in the samples. These antioxidants included vitamin E, retinol and carotenoids (lutein, zeaxanthin, anhydrolutein, beta-cryptoxanthin and beta-carotene), measured by high performance liquid chromatography (HPLC) in the laboratory of Dr. Kevin McGraw at Arizona State University. Refer to Giraudeau et al. for HPLC protocol followed (2013).

Statistical analysis. Data were analyzed using one-way ANOVA. When significance was indicated, Dunn's or Tukey post hoc analyses were used. Data are expressed as means ± SEM. Data for glucose, % glycation, retinol, anhydrolutein and uric acid were normally distributed and passed equal variance testing. Data for vitamin E, lutein and zeaxanthin were not normally distributed and were log transformed to approximate a normal distribution before analyzing using one-way ANOVA. The data for the carotenoids beta-cryptoxanthin and beta-carotene were also not normally distributed, however, because several values were 0.0, the data could not be log transformed, therefore was analyzed using Kruskal-Wallis one-way ANOVA on ranks. All statistical analyses were performed using SigmaPlot 10.0/SigmaStat 3.0. A p-value of ≤ 0.05 was considered statistically significant for all comparisons.

CHAPTER 4

RESULTS

Measurement of serum glucose levels. Serum glucose levels were similar between all birds (in mM/L: BE 20.05 ± 0.81; RTH 22.24 ± 0.93; BO 19.93 ± 1.57; GHO 21.52 ± 1.38) and were not significantly different ($p = 0.387$; see Figure 3A).

Measurement of % protein glycation. Statistical analyses showed significant between group differences in percent protein glycation. Glycation was significantly higher ($p < 0.001$) in BE (23.67 ± 1.90%) and BO (24.28 ± 1.43%) compared to RTH (14.31 ± 0.63%). Percent glycation for GHO was 19.12 ± 1.82% (see Figure 3B). For comparison, the percent albumin glycation for the chicken sample was 11.79%.

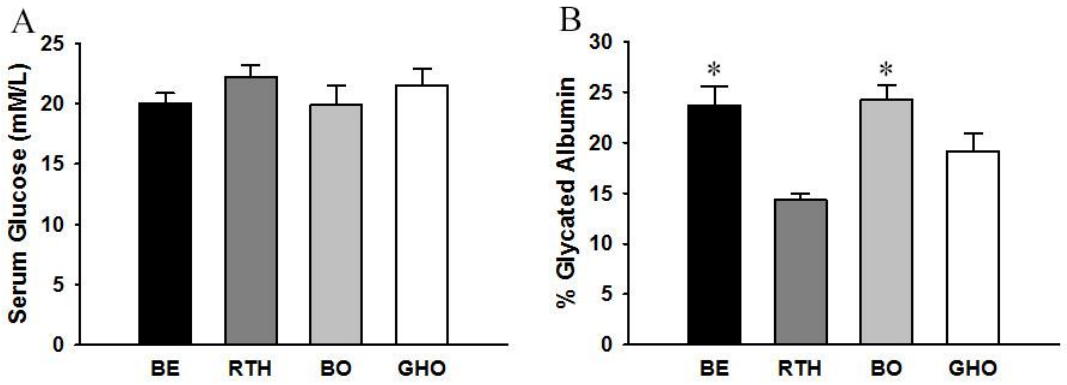

Fig. 3 Comparison of glucose and % albumin glycation levels. (A) Serum glucose levels. Levels were similar between all species ($p = 0.387$). (B) BE and BO both had significantly higher levels of protein glycation compared to RTH; * $p < 0.001$

Measurement of vasoprotective factors. Uric acid levels were not significantly different ($p = 0.355$) between species (in mg/dL: BE 9.10 ± 1.30; RTH 10.05 ± 1.24; BO 12.95 ± 2.40; GHO 11.24 ± 1.07). Vitamin E and retinol levels were also not significantly different ($p = 0.687$ and $p = 0.06$, respectively) between species (in mg/dL: BE 6.60 ± 0.68, 5.40 ± 0.83; RTH 6.67 ± 0.52; 7.94 ± 0.47; BO 7.21 ± 0.15, 5.55 ± 1.01; GHO 7.22

± 0.52, 6.70 ± 0.70). Of the carotenoids tested, beta-cryptoxanthin and beta-carotene levels were significantly greater in BE (0.59 ± 0.17; 0.41 ± 0.08 μg/ml) compared to GHO (0.02 ± 0.02; 0 μg/ml; p = 0.004) and RTH (0.02 ± 0.02; 0.01 ± 0.01 μg/ml; p = 0.001). BE and BO had significantly greater (p = 0.001) levels of lutein (6.38 ± 0.75 μg/ml; 14.54 ± 4.23 μg/ml) compared to GHO (1.96 ± 0.50 μg/ml) and RTH (2.81 ± 0.93 μg/ml). Anhydrolutein and zeaxanthin levels were not significantly different (p = 0.054 and p = 0.084, respectively) between the species (in μg/ml: BE 1.83 ± 0.51, 2.21 ± 0.50; RTH 0.82 ± 0.16, 1.51 ± 0.79; BO 1.49 ± 0.65, 6.63 ± 2.48; GHO 0.34 ± 0.095, 1.30 ± 0.26). See figure 4 (A-H) for a summary of the results.

Results for the chicken sample were as follows: uric acid 6.53 mg/dL; vitamin E 3.01 μg/ml; retinol 4.34 μg/ml; lutein 9.81 μg/ml; zeaxanthin 3.43 μg/ml; anhydrolutein 0.30 μg/ml; beta-cryptoxanthin 0.20 μg/ml; beta-carotene 0.04 μg/ml.

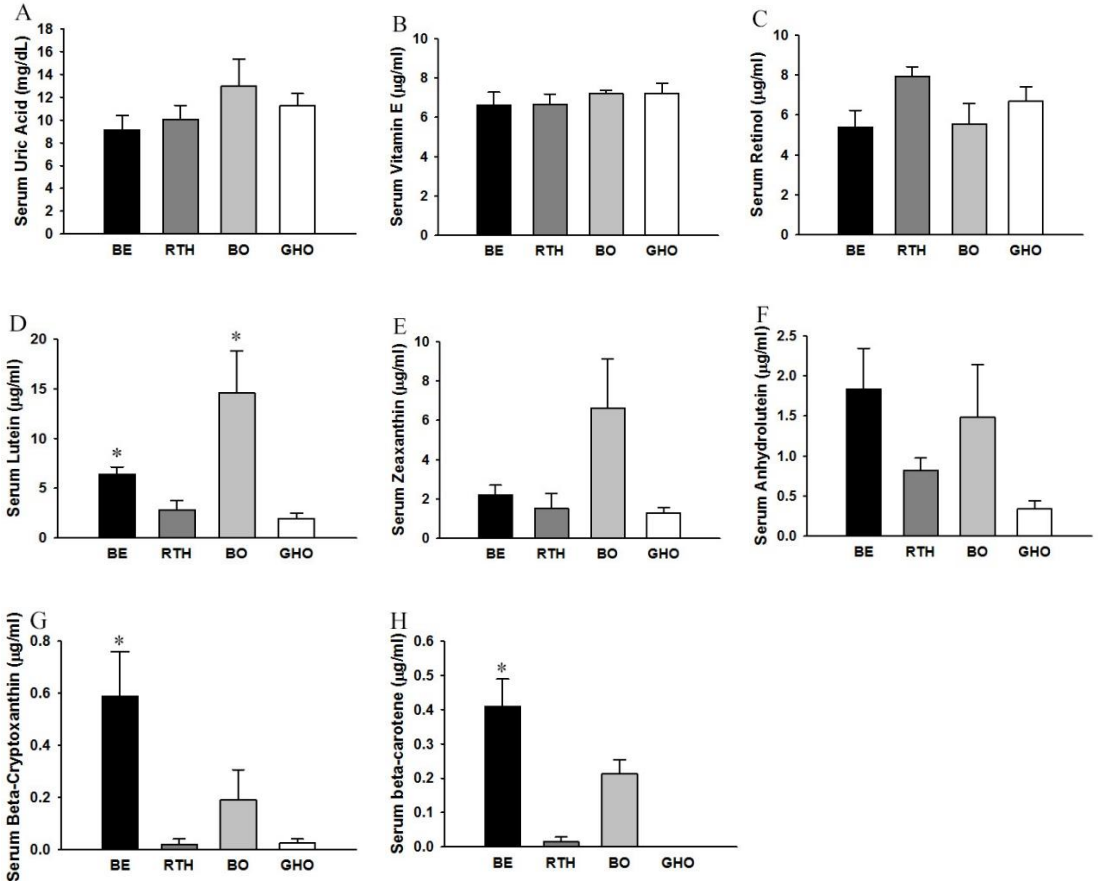

Fig. 4 Levels of vasoprotective factors. (A) Uric acid levels were not significantly different between species (p = 0.355). (B) Vitamin E levels were not significantly different between species (p = 0.589). (C) Retinol levels were not significantly different between species (p = 0.06). (D) Lutein levels were significantly greater in BO compared to GHO (*p = 0.008). (E) Zeaxanthin levels were not significantly different between species (p = 0.171). (F) Anhydrolutein levels were not significantly different between species (p = 0.054). (G) Beta-cryptoxanthin levels were significantly greater in BE compared to both RTH and GHO (*p = 0.004). (H) Beta-carotene levels were significantly greater in BE compared to RTH and GHO (*p = 0.001)

CHAPTER 5

DISCUSSION

Discussion of results. This study evaluated glucose, percent albumin glycation and several antioxidant levels in birds of prey with the aim of comparing the results to what has been observed in other species. Specifically, interest is in evaluating differences in these parameters which may exist between species that have vastly different diets.

The serum glucose levels for the birds of prey in this study ranged from 15.7 – 24.6 mM/L (282.6 – 442.8 mg/dL), which is similar to previously published values (O'Donnell, Garbett & Morzenti, 1978). Published chicken plasma glucose levels are reported between 218 – 264 mg/dL (O'Donnell, Garbett & Morzenti, 1978; Rendell et al., 1985). While both values are well above levels that would be considered normal in humans, the birds of prey have higher glucose concentrations than chickens. Diet is thought to play a major role in this difference; a carnivorous diet is low in carbohydrates and spurs a continuous state of gluconeogenesis that maintains high plasma glucose concentrations for an ever ready supply of glucose (O'Donnell, Garbett & Morzenti, 1978). Research indicates carnivores are less adaptable to carbohydrate-rich diets because of an inability to down-regulate gluconeogenesis even in the fed state (Myers & Klasing, 1999).

No studies to date have evaluated protein glycation in birds of prey. Therefore, this study is the first to measure albumin glycation in serum samples from four different species of birds of prey. The first hypothesis of this study stating that native protein glycation would be higher in birds of prey as compared to chickens due to their high protein, high fat diet with limited ingestion of antioxidants is supported by the results.

Rendell et al. reported a 2.35 ± 0.17% for chickens compared to 1.50 ± 0.05% in humans. This is interesting given hemoglobin, other glycated proteins and AGEs studied in birds (chickens, ducks, turkeys and hummingbirds) are much lower than what is found in humans, meaning although albumin glycation may be higher in chickens compared to humans, total overall glycation and AGE formation in birds is lower than in humans despite the naturally high glucose concentrations measured for these birds (Holmes, Fluckiger & Austad, 2001). In non-diabetic humans, albumin glycation can be between 1-10% *in vivo*, however one study reported a mean of 13.4 ± 2.3% in healthy individuals (Rondeau & Bourdon, 2011). The results of the present study show albumin glycation levels in bald eagles (23.67 ± 1.9%), barred owls (24.28 ± 1.43%) and great horned owls (19.12 ± 1.82%) that are above this range and, in fact, are comparable to what is seen in diabetic humans; reported means for type 1 diabetics are 25.8 ± 5.6 % and for type 2 diabetics 23.1 ± 4.4% (Rondeau & Bourdon, 2011). Interestingly, statistical analysis showed significant between species differences in the birds of prey. Both bald eagles and barred owls showed greater glycation than red-tailed hawks. Red-tailed hawks had a significantly lower level of albumin glycation (14.3 ± 0.63%), despite having the highest mean glucose levels (22.24 mM/L), compared to bald eagles and barred owls, and was similar to what has been reported in non-diabetic humans. Species variation in albumin glycation may be due to differences in albumin turnover rates; some research has shown that glycated albumin is more quickly degraded than unmodified albumin in rabbits and dogs (Kallner, 1990; Morris & Preddy, 1986). If red-tailed hawks turn over glycated albumin more quickly than the other birds of prey species, that might explain the significantly lower glycation levels compared to bald eagles and barred owls.

Alternatively, it is possible that the albumin protein may be folded differently in red-tailed hawks thus potentially shielding lysine residues from glycation or that red-tailed hawks have fewer lysine residues available for glycation. Identification of the albumin sequence and three-dimensional structure for the species examined in the current study was outside the scope of this thesis. However, a comparison of the protein sequences for humans, chickens and several other species of birds including one bird of prey is included in Appendix F. From this simple comparison, it is evident that the barn owl albumin does indeed have two variations in which a lysine residue typically expressed and known to be glycated in human samples are missing from the owl sequence (Lys 199 and Lys 519 (shown as Lys 543 in the alignment program); Anguizola et al., 2013). Thus it is possible that the red-tailed hawk albumin has similar and perhaps additional variations.

Protein glycation is promoted by oxidative stress. Also, the formation of AGEs involves oxidative steps and the interaction of AGEs with their receptors, such as RAGE, causes oxidative stress and damage. Thus, antioxidants play an important role in limiting and quenching oxidative damage. One such endogenous antioxidant that may help protect birds from oxidative damage is uric acid (Vleck, Haussmann & Vleck, 2007). Typical levels of uric acid in birds are 3.6 – 9 mg/dL (Vleck & Vleck, 2002), however, carnivorous birds have higher reference values for uric acid than other birds (Harr, 2002). The results from the present study confirm the higher reference values for uric acid in birds of prey as values range from 5.5 – 17.3 mg/dL. Birds of prey have higher levels of uric acid owing to a diet that is high in protein as increased protein metabolism and catabolism produces higher levels of uric acid. Machin et al. found that chickens fed a

high protein diet exhibited increased levels of uric acid compared to chickens that ate a low or moderate protein diet (2004). Since levels of uric acid are influenced by the amount of protein in the diet, it would be expected that birds of prey, which consume a high protein diet, would have high serum uric acid levels. Indeed, the results of the present study support the second hypothesis that uric acid levels would higher in birds of prey than chickens due to higher ingestion and utilization of protein for energy.

High levels of uric acid do not appear to be pathological to birds. For humans, uric acid is similarly an antioxidant. However, levels of uric acid above 7.2 mg/dL are pro-oxidant and can cause gout in humans (Ottum & Mistry, 2015; So & Thorens, 2010). Increased uric acid levels in diabetics may contribute to diabetic kidney disease (American Diabetes Association, 2014) and are associated with increased risk for stroke (Engstrom et al., 2002), atherosclerosis and hypertension (Ottum & Mistry, 2015). Insulin resistant individuals experience abnormal uric acid metabolism (Wilcox, 2005). A diet high in red meat, fried food, sweetened beverages and alcohol promotes elevated uric acid levels, while a diet rich in fruits, vegetables, nuts, seeds and vitamin C is associated with lower levels of uric acid (Ottum & Mistry, 2015). If uric acid retains its antioxidant capabilities even at high levels in birds, this may help explain the lower rates of oxidative stress and protein glycation observed despite the naturally high glucose concentrations.

Vitamin E is the collective name for a group of lipid soluble vitamins and antioxidants produced by plants that must be obtained from the diet (Falk & Munne-Bosch, 2010; Hidiroglou et al., 1992). Plant oils from grains, nuts, seeds and foods they are produced with are the primary source of this vitamin in the human diet, however, milk, eggs and fatty tissues of animals contain small amounts as well (Bramley et al.,

2000; Gropper & Smith, 2013). In addition to its antioxidant properties, vitamin E has been shown to help prevent glycation in vitro (Vinson & Howard, 1996) and to reduce protein glycation in diabetics (Ceriello et al., 1991). It has been reported vitamin E levels are higher in birds than in mammals, possibly due to higher lipid levels in the plasma (Schweigert et al., 1991). For example, mourning doves have been shown to have higher levels of vitamin E than rats (Smith et al., 2011). It has also been reported that birds of prey have higher levels of α-tocopherol, an important vitamin E compound, than herbivorous birds like chickens (Muller et al., 2011), although normal α-tocopherol levels are unknown (Calle, 1989). Research that has measured plasma α-tocopherol levels in captive birds of prey has shown a great deal of variability ranging from 1.95 – 40.7 μg.ml (Schweigert et al., 1991). Another study showed captive raptors supplemented with vitamin E reached plasma α-tocopherol levels between 12.3 – 849 μg/ml (Calle et al., 1989) whereas a study of 20 free-living bald eagles reported α-tocopherol levels of 24 μg/ml or more (Dierenfeld, 1989). Species-specific differences may play a role in the varying levels of plasma vitamin E observed in birds (Calle 1989), however, the birds of prey in this study had similar mean vitamin E levels (bald eagle 6.60 μg/ml, red-tailed hawk 6.67 μg/ml, barred owl 7.21 μg/ml and great horned owl 7.21 μg/ml) and were not significantly different from each other. The bald eagle mean value is less than what Dierenfeld reported for free-living bald eagles, but the difference may be due to diets obtained in captivity which were similar to all birds in the current study versus variations in food availability in the wild.

Retinol is one of a group of compounds that comprise vitamin A. It can be obtained from animal sources in the diet, such as liver, dairy and eggs, or from certain

carotenoids from plant sources, such as colorful fruits and vegetables, which are converted to retinol during digestion and absorption (Gropper & Smith, 2013). Some research has shown retinol levels are higher in birds and carnivores compared to non-carnivorous mammals (Schweigert et al., 1991). For example, Smith et al. (2011) found mourning doves had significantly higher levels of retinol than rats while Schweigert et al. (1991) found bats had only trace amounts. Birds of prey and carnivorous mammals may have higher levels of retinol due to diet; liver and other organs from prey are good sources of the vitamin, in fact, captive carnivores fed an all-meat diet without liver or supplementation will develop vitamin A deficiency diseases (Ghebremeskel & Williams, 1988; Morishita, Aye & Brooks, 1997). Schweigert et al. found chickens had a retinol mean value of 0.797 ± 0.214 μg/ml (1991). The chicken sampled in this study had a value of 4.34 μg/ml. While references for the species in this study are not available in the literature, Wallace et al. found Humboldt penguins had values ranging from 0.352 – 2.049 μg/ml (1996). The retinol levels measured in this study are greater (3.13 – 10.59 μg/ml) than these referenced values. A study of captive Psittacine birds found that retinol levels, as well as vitamin E levels, vary between subclass and genera (Torregrossa, 2005), therefore wide inter- and intraspecies variation in retinol levels can be expected and may explain the variation noted here.

Carotenoids are only synthesized by plants and must come from the diet (Brush, 1990). These compounds are important to birds for overall health, breeding and coloration (McGraw, 2006). Some carotenoids can be converted to retinol; both beta-carotene and beta-cryptoxanthin are precursors to vitamin A (Gropper & Smith, 2013). An important physiological function of carotenoids is to quench free radicals and prevent

oxidative damage (Gropper & Smith, 2013). Little is known about the dietary requirements or serum plasma levels of carotenoids in birds of prey (Blanco et al., 2013). Research in penguins, pelicans, gulls and terns have shown lutein and zeaxanthin to be the major carotenoids found in serum (Slifka, 1999). Wallace et al. found the greatest circulating carotenoid in Humboldt penguins was lutein (1996). Based on the results of this study, the same is true for bald eagles, red-tailed hawks, barred owls, great horned owls, and the chicken sample, with all species showing lutein as the most abundant carotenoid, followed by zeaxanthin. Although lutein was the most abundant carotenoid, there was high variability in the mean values between the species examined in the current study with bald eagles (6.38 ± 0.75 μg/ml) and barred owls (14.542 ± 8.461 μg/ml) having significantly greater levels than great horned owls (1.962 ± 1.108 μg/ml) and red-tailed hawks (2.81 ± 0.93 μg/ml). Bald eagles had significantly greater levels of beta-carotene and beta-cryptoxanthin than both red-tailed hawks and great horned owls. Due to the high variability in carotenoid levels between the birds of prey species and the chicken sample, the third hypothesis that carotenoid and antioxidant levels will be lower in birds of prey than in chickens due to low ingestion of antioxidants is not supported.

Since carotenoids must come from the diet, dietary preference affects levels of carotenoids in serum. However, other factors also affect carotenoid levels such as type of species, sex, age and seasonal variations in physiologic state and food availability (Blanco et al., 2013; Cohen, McGraw & Robinson, 2009; Wallace et al., 1996). Differences also exist in carotenoid levels between captive and wild birds; Andean condors living in captivity fed an all-meat diet had carotenoid levels 13% lower than their wild counterparts (Negro et al., 2002). It is suggested wild birds can forage vegetal matter

or herbivore excrement to meet carotenoid needs, whereas captive birds do not have this option (Blanco et al., 2014; Negro et al., 2002). Still more differences exist in carotenoid absorption and metabolism between species; exact biochemical mechanisms need to be elucidated (Blanco et al., 2013; Blanco et al., 2014). Since the birds of prey in this study were fed the same diet, lived in the same location and had blood drawn within the same time period, differences in biochemical parameters (carotenoid absorption, enzyme differences, different biological needs) may explain the differences in carotenoid levels observed.

Strengths and Limitations. This study had several strengths. All serum samples from birds of prey came from animals housed at the same facility. Thus, the diet and life history of the birds was known. Percent glycation was measured using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) which can measure both glycated and doubly glycated albumin allowing for more accurate measurements of total percent glycated albumin. Additionally, carotenoids and vitamins A and E were measured using a well-documented method for avian samples allowing for between species comparisons.

Despite these strengths, some limitations exist. The study only collected serum samples from the animals, thus the results cannot be extrapolated to the whole animal or other birds, animals or humans. The use of serum from captive birds, as opposed to wild birds, is another limitation. Daily activities and life histories of captive birds differ from those of wild birds, thus the findings cannot be extrapolated to wild birds. Another limitation was not having the ability to manipulate the diet of the birds to determine a direct link between dietary modifications and glycation. Also, while the diet of the birds

of prey consisted of small animals, it may not be representative of the type and timing of feedings the birds of prey would experience in the wild. Some evidence suggests wild birds will ingest unusual food sources when driven by nutrient need, however, this is not an option for captive birds. Because of inherent physiological differences between mammals and birds, findings from this study may not directly influence recommendations for humans, but may be used as a guide for further research and investigation.

CHAPTER 6

CONCLUSIONS AND APPLICATIONS

The results of this study have shown that birds of prey have metabolic similarities to diabetic humans. Both populations have high serum glucose concentrations. Birds of prey are more sensitive to insulin (unlike chickens) yet produce smaller amounts than healthy humans, a condition that mimics insulin resistance in diabetic humans. In this study bald eagles, barred owls and great horned owls exhibited similar levels of albumin glycation to what has been found in diabetic humans. For humans, chronically high serum glucose concentrations and insulin resistance lead to the development of diabetes, with high levels of protein glycation leading to the formation of AGEs. For birds of prey, however, this metabolic state is natural and they remain healthy and pathology-free. These similarities make birds of prey an excellent model of diabetes.

Even more intriguing are the results of this study that showed that while red-tailed hawks had the highest serum glucose levels, they had the lowest levels of albumin glycation. One possible explanation is that red-tailed hawks turn over glycated albumin more quickly than the other species in the study. Another explanation may be that the structure of albumin varies among the other species and humans. The protein may be folded differently in red-tailed hawks, thus shielding lysine residues from glycation. Also, it is possible that red-tailed hawks have fewer lysine residues available for glycation than the other species. The comparison of proteins sequences between humans, chickens and other birds, including barn owls, showed that owl albumin has variations in which lysine residues typically expressed and known to be glycated in human samples are missing

from the owl sequence. More research is needed to investigate the structural differences in albumin between bird of prey species and humans.

Since protein glycation is greatly affected by, and also produces oxidative stress, it is important to research factors that prevent oxidative damage from occurring. Protein glycation, AGEs and ROS can form a vicious cycle, with each promoting the other. This study evaluated the levels of certain known antioxidants. Antioxidants are of great interest because they quench ROS and thus limit oxidative damage. Birds of prey, which consume a high protein diet, have higher reference values for uric acid and the results of this study supported those values. The other antioxidants evaluated in this study – vitamin E, retinol and several carotenoids – are an interesting area of research in that these compounds are provided by the diet, thus differences in dietary composition affect levels observed. Direct comparison of each antioxidant between species and other birds and mammals is difficult given that normal levels have not been established for most species. Further complicating the issue within animal research are the differences in diet and life histories between captive and wild animals. More research is needed to investigate optimal levels of antioxidants in both captive and wild birds of prey in order to properly evaluate how a carnivorous diet affects antioxidant levels and protein glycation.

This research has significant human relevance. Studying birds of prey and how these species avoid diabetes and diabetic complications despite having high levels of plasma glucose while consuming a high protein diet could generate ideas for new research into treatment options for the millions of people who are already afflicted with diabetes and the complications caused by protein glycation and AGEs. Understanding the underlying mechanisms that protect birds from developing the complications associated

with diabetes could help develop new treatments and/or drugs that prevent glycation and thus the damaging effects of AGEs. Research into how specific diets affect protein glycation is of interest, given that dietary interventions can be a simple and cost-effective treatment.

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APPENDIX A

THE UNIVERSITY OF MINNESOTA'S INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IUCAC) APPROVAL

Protocol Title : Studies on avian resistance to protein glycation
Protocol Type : IACUC
Approval Period : 12/01/2014-11/30/2017
Important Note : **This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.**

*** Personnel Information ***

Principal Investigator (PI)

Name*
Redig, Patrick

Internet ID/x.500
redig001

Employee/Student ID
0831133

Email*
redig001@umn.edu

Phone
612/624-4969

Cell Phone
651/295-2106

Pager

Fax

Mailing Address
Raptor Center
GRaptCtr
6108A
1920 Fitch Ave
St Paul
MN
55108

City / State / Zip
St Paul / MN / 55108

Department Name
Veterinary Clinical Sciences

Dept ID
11652

Job Title
Professor

Job Code Group
Faculty

Job Code
9401

Physical Address for PI Lab

Works with Animals* Y

Is this person experienced with all species that he/she will use and all procedures he/she will be performing on this protocol?* Y

Indicate with which procedures or species this person is not experienced and how they will be trained.

Click here for additional information about training.

Course Title	Course ID	Required / Recommended	Completion Date
IACUC Training Certification	IC1000	Required for approval if working with animals	2003-06-01 00:00:00.0

If you have completed training that is not indicated above, please describe training you received (include name of course provider, course number and brief description of coursework):

Alternate Submitter

Secondary Investigator

Additional Staff

APPENDIX B

ARIZONA STATE UNIVERSTIY'S INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) APPROVAL

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
660 South Mill Avenue, Suite 315
Tempe, Arizona 85287-6111
Phone: (480) 965-4387 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number:	**15-1398R**
Protocol Title:	**Regulation of Glucose Homeostasis and Vascular Reactivity in Birds**
Principal Investigator:	**Karen Sweazea**
Date of Action:	**11/20/2014**

The animal protocol review was considered by the Committee and the following decisions were made:

The protocol was approved as presented.

If you have not already done so, documentation of Level III Training (i.e., procedure-specific training) will need to be provided to the IACUC office before participants can perform procedures independently. For more information on Level III requirements see https://researchintegrity.asu.edu/training/animals/levelthree.

Total # of Animals:	**434**	
Species:	**Birds**	**Pain Level: C-264; D-170**
Protocol Approval Period:	**11/20/2014 – 11/19/2017**	
Sponsor:	**N/A**	
ASU Proposal/Award #:	**N/A**	
Title:	**N/A**	

Signature: Augustowski for C. Johnson Date: 11/26/2014
IACUC Chair or Designee

Cc: IACUC Office
IACUC Chair

APPENDIX C

PROTOCOL FOR GLUCOSE COLORIMETRIC ASSAY KIT

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Glucose Assay Standard - (Item No. 10010098)**

   The vial contains 300 µl of 1,000 mg/dl glucose. It is ready to use as supplied to prepare the standard curve. Sufficient Standard is provided to prepare four standard curves.

2. **Sodium Phosphate Assay Buffer - (Item No. 700003)**

   The vial contains 10 ml of 250 mM sodium phosphate, pH 7.2. Dilute the contents of the vial with 40 ml of HPLC-grade water. This solution is used to prepare the diluted Glucose Standards and for the dilution of the Enzyme Mixture. The diluted Buffer is stable for three months at 4°C.

3. **Glucose Colorimetric Enzyme Mixture - (Item No. 10010100)**

   The vial contains a lyophilized enzyme mixture. Reconstitute 1 vial with 6 ml of diluted Assay Buffer and mix well. This reconstituted solution is now ready to use in the assay. The reconstituted solution is stable for at least one hour when stored at 4°C. One vial of the Enzyme Mixture is sufficient to evaluate 60 wells.

Sample Preparation

Plasma

Typically, normal human plasma has glucose concentrations in the range of 70-110 mg/dl.[5]

1. Collect blood using an anticoagulant such as heparin, EDTA, or citrate.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice. If not assaying the same day, freeze at -80°C. The plasma sample will be stable for one month while stored at -80°C.
3. Dilute plasma 1:5 with diluted Assay Buffer before assaying.

Serum

Typically, normal human serum has glucose concentrations in the range of 70-110 mg/dl.[5]

1. Collect blood without using an anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The serum sample will be stable for one month while stored at -80°C.
4. Dilute serum 1:5 with diluted Assay Buffer before assaying.

Urine

Typically, normal human urine has glucose concentrations in the range of 1-15 mg/dl.[5]

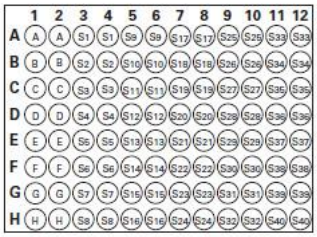
1. Urine does not require any special treatments. If not assaying the same day, freeze at -80°C.

NOTE: Glucose values from urine samples can be standardized using Cayman's Creatinine (urinary) Assay Kit (Item No. 500701).

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of glucose standards and samples to be measured in duplicate is given below in Figure 2, below. We suggest you record the contents of each well on the template sheet provided (see page 18).



A-H = Standards
S1-S40 = Sample wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 200 µl in all wells.
- The incubation temperature is 37°C.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the standards and samples be assayed at least in duplicate.
- Monitor the absorbance at 500-520 nm using a plate reader.

Standard Preparation

Dilute 50 µl of the 1,000 mg/dl Glucose Standard with 450 µl of diluted Assay Buffer to make a 100 mg/dl stock. Take eight clean 12 x 75 mm glass test tubes or polystyrene tubes and label them A-H. Add the amount of Glucose Standard and Assay Buffer to each tube as described in Table 1. The diluted Glucose Standards are stable for two hours at room temperature.

Tube	Glucose Stock (µl) (100 mg/dl)	Assay Buffer (µl)	Glucose Concentration (mg/dl)
A	0	200	0
B	5	195	2.5
C	10	190	5
D	15	185	7.5
E	20	180	10
F	30	170	15
G	40	160	20
H	50	150	25

Table 1. Glucose standards to be assayed along with samples.

Performing the Assay

1. **Glucose Standard wells** - Add 85 µl of diluted Assay Buffer and 15 µl of each Standard (tubes A-H) to two wells (see suggested plate configuration, Figure 2, page 10).
2. **Sample wells** - Add 85 µl of diluted Assay Buffer and 15 µl of sample to two wells.
3. Initiate the reaction by adding 100 µl of Enzyme Mixture to all standard and sample wells.
4. Cover the plate with the plate cover and incubate for 10 minutes at 37°C.
5. Remove the plate cover and read the absorbance at 500-520 nm using a plate reader.

ANALYSIS

Calculations

1. Calculate the average absorbance of each standard and sample.
2. Subtract the absorbance value of the standard A (0 mg/dl) from itself and all other values (both standards and samples). This is the corrected absorbance.
3. Plot the corrected absorbance values (from step 2 above) of each standard as a function of the concentration of glucose (see Table 1, page 12).
4. Calculate the concentration of glucose for each sample from the standard curve. An example of the glucose standard curve is shown in Figure 3, see page 15.

$$\text{Glucose (mg/dL)} = \left[\frac{\text{(Corrected absorbance) - (y-intercept)}}{\text{Slope}}\right] \times \text{dilution}$$

Performance Characteristics

Precision:

When a series of thirty-six human serum and urine samples were assayed on the same day, the intra-assay coefficient of variation was 4.6% and 8.1%, respectively. When a series of thirty-six human serum and urine samples were assayed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 1.7% and 11.3%, respectively.

Assay Range:

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0-25 mg/dl.

Representative Glucose Standard Curve

The standard curve presented here is an example of the data typically provided with this kit; however, your results will not be identical to these. You must run a new standard curve - do not use these to determine the values of your samples.

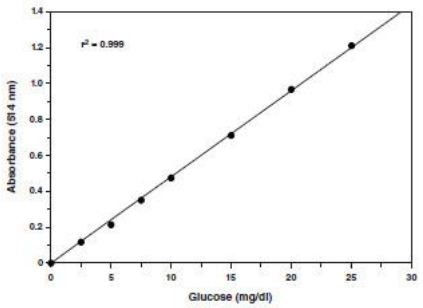


Figure 3. Glucose standard curve

APPENDIX D

PROTOCOL FOR LC-ESI-MS ANALYSIS OF BIRD OF PREY AND CHICKEN SERUM SAMPLES FOR PERCENT GLYCATION

LC-ESI-MS Machine Preparation:

1. Turn MS from ‘stand-by’ mode to ‘operate’ mode
2. Change loading flow from 3µl/min to 10µl/min and change micro flow from 0.105µl/min to 3µl/min
3. Screw in the connection between the LC and MS
4. Clean source using methanol and water; clean needle using methanol and water

Sample Preparation:

1. Prepare 5ml 0.1% TFA by combining 4.95ml distilled H_2O with 50µl 10% TFA stock solution
2. Remove sample from -80°C
3. Centrifuge sample for 60 seconds at 14 RPM
4. Prepare 1000:1 dilution of raptor sample by combining 500µl 0.1% TFA with 0.5µl sample. Vortex diluted sample
5. Load 25µl of the diluted sample into one well of the 96-well plate
6. Load 50µl of 0.1% TFA into a separate well of the 96-well plate
7. Load 96-well plate onto the plate-holder of the LC-MS
8. Acquisition the two wells as follows:
    a. Sample well-
        i. Injections: 1
        ii. Amount: 10.000µl
        iii. LC Method Part: alb apoai captrap step grad 35 45 80
        iv. Autosampler Method Part: Standard
        v. MS Acquisition Method Part: 160605 has optimized for 3 mclmin
    b. Solvent well-
        i. Injections: 1
        ii. Amount: 10.000µl
        iii. LC Method Part: alb apoai captrap step grad 35 45 80
        iv. Autosampler Method Part: Standard
        v. MS Acquisition Method Part: 160605 has optimized for 3 mclmin

Spectra Analysis (Albumin):

1. Open Spectra in Compass DataAnalysis
2. Run method: Albumin
3. Uncheck BPC and All MS
4. Edit Chromatogram to add Total Ion Chromatogram (TIC)
5. Average raw spectra across the approximate 0.5 min timeframe of sample elution
6. Copy to compound spectra
7. Baseline subtraction (0.7)
8. Charge Deconvolution
9. Record the mass (m/z) and counts for the relevant peaks for samples (native albumin, glycated albumin, and doubly glycated albumin)
10. Calculate Percent Glycated Albumin for each spectrum using the appropriate counts
11. Save spectra

APPENDIX E

PROTOCOL FOR URIC ACID ASSAY

QuantiChrom™ Uric Acid Assay Kit (DIUA-250)

Quantitative Colorimetric Uric Acid Determination at 590nm

DESCRIPTION

Uric acid is the waste product produced from the degradation of purines. In healthy human, uric acid is filtered and removed from the blood by the kidneys and excreted into urine. Because a number of kidney diseases are known to affect uric acid levels, uric acid determination is thus important and useful in diagnosing and evaluating kidney diseases. For example, when uric acid is present in the blood at abnormally high levels, it tends to crystallize in body joints, resulting in gout, a very painful inflammatory condition. Increased levels of uric acid are also known to be associated with uremia, leukemia and pneumonia.

Simple, direct and automation-ready procedures for measuring uric acid concentration in blood are becoming popular in Research and Drug Discovery. BioAssay Systems' uric acid assay kit is designed to measure uric acid directly in serum without any pretreatment. The improved method utilizes 2,4,6-tripyridyl-s-triazine that forms a blue colored complex specifically with iron in the presence of uric acid. The intensity of the color, measured at 590nm, is directly proportional to the uric acid concentration in the serum. The optimized formulation substantially reduces interference by substances in the raw samples.

KEY FEATURES

Sensitive and accurate. Use 5 µL samples. Linear detection range 0.22 mg/dL (13µM) to 30 mg/dL (1785 µM) uric acid in 96-well plate assay.
Simple and high-throughput. The procedure involves addition of a single working reagent and incubation for 30 min. Can be readily automated as a high-throughput assay in 96-well plates for thousands of samples per day.
Improved reagent stability and versatility. The optimized formulation has greatly enhanced reagent and signal stability. Cuvet or 96-well plate assay.
Low interference in biological samples. No pretreatments are needed. Assays can be directly performed on serum samples.

APPLICATIONS:

Direct Assays: uric acid in serum, plasma, urine and other biological samples.
Drug Discovery/Pharmacology: effects of drugs on uric acid metabolism.

KIT CONTENTS (250 tests in 96-well plates)

Reagent A: 50 mL **Reagent B: 6 mL**
Reagent C: 6 mL **Standard: 1 mL 10 mg/dL uric acid**
Blank Control: 1 mL

Storage conditions. The kit is shipped at room temperature. Store reagents at 4 °C, standard and blank control at -20 °C. Shelf life: 12 months after receipt.

Precautions: reagents are for research use only. Normal precautions for laboratory reagents should be exercised while using the reagents. Please refer to Material Safety Data Sheet for detailed information.

PROCEDURES

Reagent Preparation: shake Reagent C before use. Prepare enough working reagent by mixing 10 volumes of Reagent A, 1 volume Reagent B and 1 volume Reagent C. Fresh reconstitution is recommended. Equilibrate to room temperature before assay. Metal chelators (e.g. EDTA) interfere with this assay and should be avoided.

Procedure using 96-well plate:

1. Set up standards and samples. Transfer 5 µL Blank, Standard and samples in duplicate wells of a clear bottom 96-well plate.
2. Add 200 µL working reagent and tap lightly to mix.
3. Incubate 30 min at room temperature and read optical density at 510-630nm (peak absorbance at 590nm).

Procedure using cuvette:

1. Set up test tubes labeled Blank, Standard, Samples. Transfer 20 µL Blank, Standard and samples to appropriately labeled tubes.
2. Add 1000 µL working reagent and tap lightly to mix.
3. Incubate 30 min at room temperature and read optical density at 590nm (510nm-630nm).

CALCULATION

The uric acid concentration of Sample is calculated as

$$= \frac{OD_{SAMPLE} - OD_{BLANK}}{OD_{STANDARD} - OD_{BLANK}} \times 10 \text{ (mg/dL)}$$

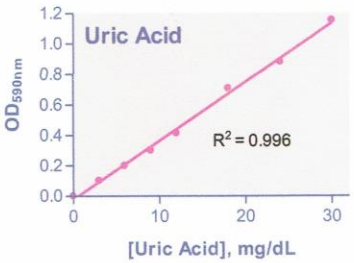
OD_{BLANK}, $OD_{STANDARD}$ and OD_{SAMPLE} are OD_{590nm} values of Blank, Standard and Sample, respectively. It is not necessary to prepare a calibration curve, because the concentration of the provided standard lies within the linear range.
Normal serum uric acid values: 1.0 to 7.0 mg/dL.
Conversions: 1 mg/dL uric acid equals 59.5 µM, 0.001% or 10 ppm.

MATERIALS REQUIRED, BUT NOT PROVIDED

Pipeting devices and accessories (e.g. 5 µL).
Procedure using 96-well plate:
Clear bottom 96-well plates (e.g. Corning Costar).
96-well plate absorbance (590nm) reader.
Procedure using cuvette:
Cuvets for measuring optical density at 510-630nm.
Spectrophotometer for measuring absorbance at 590nm.

EXAMPLES:

Samples were assayed using the 96-well protocol. The uric acid content (mg/dL) was 1.3 ± 0.1 (n = 4) for mice serum, 2.6 ± 0.0 (n = 4) for fetal bovine serum (Invitrogen), 1.4 ± 0.1 for goat serum, 1.3 ± 0.1 for rat serum, 2.9 ± 0.1 for rat plasma, 3.4 ± 0.1 for human serum and 1.4 ± 0.1 for human plasma, respectively.



Standard Curve in 96-well plate assay

PUBLICATIONS

[1]. Viel, E.C. et al (2008). Xanthine oxidase and mitochondria contribute to vascular superoxide anion generation in DOCA-salt hypertensive rats. Am J Physiol Heart Circ Physiol. 295:H281-H288.

[2]. Kamel, A. H. (2007). Conventional and planar chip sensors for potentiometric assay of uric acid in biological fluids using flow injection analysis. J Pharm Biomed Anal. 45(2):341-348.

[3]. DiSilvestro R. A. et al (2009). Pomegranate extract mouth rinsing effects on saliva measures relevant to gingivitis risk. Phytother Res. 23(8): 1123-1127.

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Tel: 510-782-9988, Fax: 510-782-1588 · Email: order@bioassaysys.com, info@bioassaysys.com

APPENDIX F

COMPARISON OF THE PROTEIN SEQUENCES FOR HUMANS, CHICKENS AND SEVERAL OTHER SPECIES OF BIRDS INCLUDING ONE BIRD OF PREY PREPARED USING THE CONSTRAINT-BASED MULTIPLE ALIGNMENT TOOL (COBALT) PROGRAM FROM NCBI (NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION)

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Human                     1   MKWVTFISLLFLFSSAYSRGVFR--RDA-HKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTC   77
Cow                       1   MKWVTFISLLLLFSSAYSRGVFR--RDT-HKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHVKLVNELTEFAKTC   77
Chicken                   1   MKWVTLISFIFLFSSATSRNLQRFARDAEHKSEIAHRYNDLKEETFKAVAMITFAQYLQRCSYEGLSKLVKDVVDLAQKC   80
Golden-collared manakin   1   MKWLTLISFIFLLSSARSRNLHRAARDADHKSQIAHRYNDLKEETFKGVAMITFAQYLQRCSYDGLSKLVKDIVDLAHKC   80
American crow             1   MKWLALISFLFLLSSARSRNLQRTARDADHKSPIAHRFKDLKEETFKAVTMITFAQYLQRCSYEGLSKLVKDVVDLAHKC   80
Hoatzin                   1   MKWVTLISFIFLFTSAASRNLQRVARDAEHKSEIAHRFNDLKEETFKAATMITFAQYLQRCSYEGLSKLVKDVVDLAHKC   80
Killdeer                  1   MKWVTLISFIFLFSSATSRNLQRVARDTEYKSEIAHRYNDLKEETFKAVTMITFAQYLQRCSYDGLSKLVKDVVDLAQKC   80
S. carmine bee-eater      1   MKWVTLISFLFLLSSARSWNLQRAARDAEHKSEIAHRYNDLKEETFKAVAMITFAQYLQRCSYDGLSNLVKDVVDLAQKC   80
Brown mesite              1   MKWVTLISFLFLLNSATSRNLQRVARGAEHKSEIAHRYEDLHEETFKAVSMITFAQYLQKCSYDGLSKLVKDVVDLAQKC   80
Sunbittern                1   MKWVTLISFIFLFNSATSRNLQRVARDTEHKSEIAHRYNDLKEETFKAVTMVTFAQYLQRCSYEGLSKLVKDVVDLAQKC   80
Rifleman                  1   MKWLTFVSLILLLSSATSRNLQRVARDADHKSEIARRYNDLKEETFKAVTMITFAQYLQKCSYDALSKLVKDVVDLAQKC   80
Adelie penguin            1   MKWVTLISFIFLLSSARSRNLQRAARDAEHKSEIAHRYNDLKEETFKAVTMITFAQYLQRCSYDGLSKLVKDVVDLAQKC   80
Emperor penguin           1   MKWVTLISFIFLFSSARSRNLQRVAREAEHKSEIAHRYNDLKEETFKAVTMITFAQYLQRCSYDGLSKLVKDVVDLAQKC   80
Red-throated loon         1   MKWVTLISFIFLFSSATSRNLQRVAREAEYKSEIAHRYNDLKEETFKAVTMITFAQYLQRCSYDGLSKLVKDVVDLAQKC   80
Crested ibis              1   MKWATLISSVFLLSSATFLNLQNLAQAADHKSEIAHRYNDLKEETFKAVALITFAQYLQRCSYEGLSNLVKNVVDLAHTC   80
Barn owl                  1   MKWVTLISFIFLLSSATSWNLERVPREAEHKSEIAHRYNDLKEETFKAVTMITFAQYLQRCSYDGLSKLVKDVVDLAQKC   80
Anna's hummingbird        1   MKWVTLISLIFLFSSAASRNLQRVARGTEHKSEIAHRYKDLKEETFKAVTLITFAQYLQRCSYEGLSKLVKNVVDLAQKC   80

Human                    78   VADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPN-LPRLVRPEVDVMCTAFHDNEE   156
Cow                      78   VADESHAGCEKSLHTLFGDELCKVASLRETYGDMADCCEKQEPERNECFLSHKDDSPD-LPKL-KPDPNTLCDEFKADEK   155
Chicken                  81   VANEDAPECSKPLPSIILDEICQVEKLRDSYGAMADCCSKADPERNECFLSFKVSQPDFVQPYQRPASDVICQEYQDNRV   160
Golden-collared manakin  81   VANEDAPECTKPLPTIFLDRICQVDQLRDSYGDMADCCGKADPERNQCFLSFRVHHPDFIPPYQRPAADVICTQYQDNRV   160
American crow            81   VANEDAPECSKALPSIFLDEICQVDKLRDSYGDMADCCGKADPERNQCFLSFKVHQPDFIPPYQRPAADVICNKYKDERV   160
Hoatzin                  81   VANEDAPECSKSLPSIFLDEICHIEKLRDSYGDMADCCSKADPERNECFLSFKTHHPDFIQPYQRPANDVICKEYQDNRM   160
Killdeer                 81   VANEDAPECTKPLPSIFLDEICQVEKLRDSYGAMADCCSKADPERNECFLSFKVPQPDFVQPYQRPAADVICKEYQDNRV   160
S. carmine bee-eater     81   VANEDAPDCTKSLPSIFLDEICQVEKLRDSYGAMADCCAKADPERNQCFLSFKVPQPDFVQPYVRPAADVICKEYQDNRV   160
Brown mesite             81   VANKDAPDCDKPMPTIFLDEICQVEKLRDSYGAMADCCSKADPERNECFLSFKVHQPDFIQPYQRPASDVVCKEYQDDRV   160
Sunbittern               81   VANEDAPDCAKSLPSIFLDEICQVEKLRDSYGAMADCCGKADPERNECFLSFKIPQPDFVQPYQRPATDVICKEYQDNRV   160
Rifleman                 81   AANEDAPECAKSLPAIFLDEICQVEKLRDSYGDMADCCAKSDPERNQCFLSFKVHQPDFIEPYQRPAADVICNEYKDNRV   160
Adelie penguin           81   VANEDAPECTKPLPSIFLDEICQVEKLRDSYGAMADCCSKADPERNECFLSFKVSQPDFVQPYQRPASDVICKEYQDNRM   160
Emperor penguin          81   VANEDAPECTKSLPSIFLDEICQVEKLRDSYGTMADCCSKADPERNECFLSFKVSQPDFVQPYQRPASDVICKEYQDNRV   160
Red-throated loon        81   VANEDAPECTKSLPSIFLDEICQVEKLRDSYGAMADCCSKADPERNECFLSFKVPQPDFIQPYQRPASDVICKEYQDNRV   160
Crested ibis             81   VANEDAPGCTKSLPAIFLDNICQVEKLRDSYGAMADCCGKADPERNECFLSFKVPLPDFVQPYQRPASDVICQEYQDNRV   160
Barn owl                 81   VANEDAPECTKSLPSIFLDEICQVEKLRDSYGAMADCCGKADPERNECFLSFKVPQPDFVQPYQRPASDVICKEYQDNRV   160
Anna's Hummingbird       81   VANEDAPECSKSLPSIFLDEICQVEKLRDSYGAMADCCGKADPDRNQCFLSFKVPQPDFVQPYQRPAADQICKEYQDNRV   160

Human                   157   TFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFK   236
Cow                     156   KFWGKYLYEIARRHPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPKIETMREKVLTSSARQRLRCASIQKFGERALK   235
Chicken                 161   SFLGHFIYSVARRHPFLYAPAILSFAVDFEHALQSCCKESDVGACLDTKEIVMREKAKGVSVKQQYFCGILKQFGDRVFQ   240
Golden-collared manakin 161   ALLGNFVYTVARRNPFLHAPAILALAAEYENALKTCCSESDIGACLDEKASAIKDRAKIIGVQQQHGCRVLEKYGERTFQ   240
American crow           161   SLLGHFIYTVARRNPFLHAPAILGLAAEYENALKSCCPESDIGACLDAKAVVIKERAKLIGVKQQHGCRILDKYGERTFQ   240
Hoatzin                 161   SLLGHFIYAVARRNPFLYAPTILSLAADYEHSLQSCCKESDVGTCLDEKAAVIKERAKKVSLKQLHSCGILKKFGERTFG   240
Killdeer                161   PFLGHFIYSVARRNPFLYAPTILSLAADYEHALQTCCAESDVGACLDEKANAIKERAKKVSVKQQYSCGILKKFGERTFK   240
S. carmine bee-eater     161   SLLGHFVYSVARRNPFLYAPTILGLAVDYEHALQSCCKETDVGACLDEKVAAIKERAKKVSVKQQYSCGVLDKFGDRTFQ   240
Brown mesite            161   SLLGHFVHSVARRNPFLYAPTIVSLAADYEHALQTCCKESDVDACLDEKATTIKEKAKKMSVKQQYVCGVRSKFGERVLQ   240
Sunbittern              161   SLLGHFIYTVARRNPFLYAPTILGLAADYEHALQSCCKESDVGTCLDEKATAIKERAKKVSVKQHYFCGVLQKFGERTFQ   240
Rifleman                161   ALLGHFVYTVARRNPFLHAPAILGLAIEYEHALQSCCSESDVGACLDEKAATIKERAKMINKKQQHGCRILEKYGERTFQ   240
Adelie penguin          161   PFLGHFIYSVARREPFLYAPTILSLAADYEHALQTCCKESDVGACLDEKAAAIKERAKKVSVKQQYSCGILKKFGERTFK   240
Emperor penguin         161   PFLGHFIYSVARREPFLYAPTILSLAADYEHALQTCCKESDVGACLDEKAAAIKERAKKVSVKQQYSCGILKKFGERTFK   240
Red-throated loon       161   SLLGHFIYSVARRNPFLYAPTILSLAADYEHALQSCCKESDVGACLDEKATAIKERAKKVSVKQQYSCGILKKFGERTFK   240
Crested ibis            161   SLLGHFIYSVARRNPFLYAPTILSLAADYEHALQSCCKETDVSACLDEKAIAIKEKAKKVSVTQQYSCGILNKFGERTFK   240
Barn owl                161   SLLGHFIYSVARRNPFLYAPTILSLAADYEHALQTCCKESDVGACLDEKAVAIKERAKKVSVKQQYSCGILKKFGERTFQ   240
Anna's Hummingbird      161   SLLGHFIYSVGRRNPFLYAPTILSLAADYEHALQVCCKENDVSACLDEKATAIKEKAKKASMKHQYSCGILKKFGERTFK   240

Human                   237   AWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAE   316
Cow                     236   AWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAE   315
Chicken                 241   ARQLIYLSQKYPKAPFSEVSKFVHDSIGVHKECCEGDMVECMDDMARMMSNLCSQQDVFSGKIKDCCEKPIVERSQCIME   320
Golden-collared manakin 241   ADKLARMSQKYPKASFAELIKMVQEVKNVYRECCEGDMVECVDDWSELMNSLCAKQDVFSSKIKPCCEKPAVERTSCIVE   320
American crow           241   ASKLVRMSQKYPQAPFPELVKMVHDVKDVYKECCEGDMVECVDDWSELVTSLCSKQDIFSSKIKPCCEKPVVERTRCIIE   320
Hoatzin                 241   ADKLAQLSQKYPKAPLPEILKILHDVKGVYKECCEGDMVECMDDRAELVVYVCSKQDILSSKIKECCEKPVVERSQCIIE   320
Killdeer                241   ADYLALLSQKYPKAPFSEILKILGDIKDIYKECCEGDMVECMDDRAELMTYICSKQDVFSSKIKHCCEKPVVERSQCIIE   320
S. carmine bee-eater     241   ANKLVALSQKYPKAPFTEIVKILKDILKVHKECCDGDMVECMDDRAEIATYMCSKQEVFSSKIKDCCEKPVVERSECIIE   320
Brown mesite            241   ADKLARLSQKYPKTPFSEILKIVSDLKGVYKECCEGDMIECMDDMAEIMTYMCSKQDVFSSKIGHCCEKPVVERSQCIIE   320
Sunbittern              241   ANMLARLSQKYPKAPFSEIIKILRGTKDVYRECCEGDMIECMDDRAELMTYICSKQDTFSSKIKDCCEKPIVERSQCIIE   320
Rifleman                241   AMKLARMSQKYPKAPFAEVVKMVHDTKDVYKECCEGDMVECVDDWSELLHSLCAKQDVFSSKIKSCCEKPVLEQTKCIIE   320
Adelie penguin          241   ADKLALLSQKYPKAPLSEMLKILHDIMGIYKECCEGDMVECMDDRAELMTYMCSKQDVFSSKIKDCCEKPVVERSQCIIE   320
Emperor penguin         241   ADKLALLSQKYPKAPLSEMLKILQHVMGIYKECCEGDMVECMDDRAELMTYMCSKQDVFSSKIKDCCEKPVVERSECIIE   320
Red-throated loon       241   ADKLALLSQKYPKAPLSEILKILQDIKGIYKECCEGDMVECMDDRAELMTYMCSKQDVFSSKIKDCCEKPVVERSQCIIE   320
Crested ibis            241   ADKLALLSQKYPKAPFSEILKLLQDIKGIYKECCEGDMVECMDDRAELMTYMCSKQDVFSSKIKHCCEKPVVERSQCIIE   320
Barn owl                241   ADKLALLGQKYPKAPFSEILKILRDIKGIYRECCEGDMVECMDDRAELMTYMCSKQEVFSSKIKHCCEKPVVERSQCIIE   320
Anna's Hummingbird      241   ADKLVKVSQKYPKAPFSEVTKILGDITGIYEECCDGDMVECMDDRAELMSYICSKQDVFSSKIKECCEKPVVEQSQCIIE   320
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Human                    317  VENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAK  396
Cow                      316  VEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECCAKDDPHACYST  395
Chicken                  321  AEFDEKPADLPSLVEKYIEDKEVCKSFEAGHDAFMAEFVYEYSRRHPEFSIQLIMRIAKGYESLLEKCCKTDNPAECYAN  400
Golden-collared manakin  321  ADFDEKPDNLPSLVEKYIQDKEVCKSYEAGHDAFLSEFVYEYSRRHPELATTVILRVAKGYETLLDKCCKTDNPAECYGH  400
American crow            321  ADFDDKPDNLPSLVEKYIQDKEVCKSYEPNHDAFLSEFVYEYSRRHPEFSTQLIMRITKGYETLLDKCCKTDNPAECYGN  400
Hoatzin                  321  ADFDDIPEDLPSLVEKYVEDKEVCKHFEANHDGFLTEFIYEYGRRHPEYSTQLILRVTKGYEELLQKCCKTDNPAECYAH  400
Killdeer                 321  ADYDDKPENLPSLVEKYVHDKEVCKSFEAGRDEFLAELVYEYSRRHPEFSTQLILRITKGYETLLEKCCKTDNPAECYGN  400
S. carmine bee-eater     321  AEFDDKPEDLPSLVEKYINDKAVCERFNAGHDEFLSEFVYDYSRSHPEFSTQLILRIAKGYETLLEKCCKTDNPAECYAN  400
Brown mesite             321  AEFDDKPENLPSLVEKYVEDKEVCKSFEAGHDAFMSEFVYEHARRHPEFSTQLILRIAKGYETLLEKCCKTDNSAECLGN  400
Sunbittern               321  ADFDDKPEDLPSLVEKYIQDKEVCKSFEADHDAYLSEFVYEYARRHPEFSTQLILRIAKGYETLLEKCCKTDDPSACYGN  400
Rifleman                 321  ADFDDKPDNLPSLVEKYVQDKEVCKSYEAGHDAFLSEFVYEYSRRHPEFSTQLIMRITKGYEALLDRCCKTDNPAECYGN  400
Adelie penguin           321  ADFDDQPEGLPSLVERYLEDKEVCKGFEAGHDAFMAEFVYEYSRRHPEFSTQLILRVSRGYETLLEKCCKTDNPPECYGN  400
Emperor penguin          321  AEFDDKPEDLPSLVERYLEDKEVCKGFEAGHDAFMAEFVYEYARRHPEFSTQLILRVSKGYEALMEKCCKTDNPPECYGN  400
Red-throated loon        321  AEFDDKPEDLPSLVERYLQDKEVCKSFEAGHDQFLAEFVYEYSRRHPEFSTQLILRVSKGYETLLEKCCKTDNPADCYGN  400
Crested ibis             321  ADYDDKPEGLPSLVEKYIESQEVCKSFEADHDAYLSEFLYEYSRRHPELSTQLILRITKGYETLLEKCCKTPNPAECYGN  400
Barn owl                 321  ADFDDKPEDLPSLVEKYIQDKEVCKSFEADHDAFLSEFVYEYSRRHPEFSTQLILRVAKGYETLLEKCCKTDNPAECYGN  400
Anna's Hummingbird       321  SDMDDKPEDLPSLAEKYIQDKEVCKSFERDHDGFLSEFLYEYSRRHPEFSSQLILRIAKGYENLLEKCCKTDNPAECYGN  400

Human                    397  VFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDY  476
Cow                      396  VFDKLKHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDY  475
Chicken                  401  AQEQLNQHIKETQDVVKTNCDLLHDHGEADFLKSILIRYTKKMPQVPTDLLLETGKKMTTIGTKCCQLPEDRRMACSEGY  480
Golden-collared manakin  401  AEEELNKHIKETQELVKTNCDLFNTHGEGDFLKGILVRYTKKMPQVSTDTLLEIGKKMTAVGAKCCSLPEEKRLACSERY  480
American crow            401  AVEDLNKHIKETEDVVKTNCELFNTHGEADFLKGILVRYTKKMPQVSTDTLLEIGKKMTAVGNKCCSLPEEKRMSCSEHY  480
Hoatzin                  401  AVDESKKHIKETQDVVKTNCELLTAHGEGDFLKGLLIRYTKKMPQVSTDTLLELGKKMTTVGTKCCQLPEARRLPCSEGY  480
Killdeer                 401  AHEELNKHIKETQDVVKTNCDILTTHGEADFLKALLIRYTKKMPQVSTDTLLEIGKKMTAVGTKCCQLPEDRRLPCSEGY  480
S. carmine bee-eater     401  AVEDLNKHIKETQDVVKTNCDFLNTHGEADFLKALLIRYTKKMPQVSTETLLEIGKKMTHVGTKCCNLPEDRRLPCSEGY  480
Brown mesite             401  AVEELNKHIKETQDVVKTNCDLLTAHGEKEFLKALLIRYTKKMPQVSTDTLLEIGKKMTAVGNKCCQLPEDRRLPCSEGY  480
Sunbittern               401  AVEELNKLIKETEDVVKTNCELLTTHGEADFLKALLIRYTKKMPQLSTDALIETGKKMTAIGNKCCTLPENKRLPCSEGY  480
Rifleman                 401  AVEELEKQVKEAQDVVNTNCELFNTHGKEDFLKGILIRYTKKMPQVSTDTLLQVGKKMTAVGAKCCSLPEKKRMACSELF  480
Adelie penguin           401  TVEMLHKPIKETQEVVKTNCELLTAQGETGFLKALLIRYTKKMPQVSTDTLIEIGKKMTAVGTKCCQLPEDRRLPCSEGY  480
Emperor penguin          401  TVEMLHKPIKETQEVVKTNCELLTTKGETDFLKALLIRYTKKMPQVSTDTLVEIGKKMSAIGTKCCQLPEDRRLPCSEGY  480
Red-throated loon        401  AQEELHKHIKETQDVVKTNCELLTTHGEPDFLKALLIRYTKKMPQVTTDTLLEIGKKMTAVGTKCCQLPEDRRLRCSEGY  480
Crested ibis             401  AVEELNKHIKETQDVVQTNCDLLTAHGEHDFLKALLIRYTKKMPQVSTETLLEIGQKMTAVGTKCCHLPKDRRLPCSEGY  480
Barn owl                 401  AVEDLNKHIEETQDVVKTNCELLTTHGEQDFLKALLIRYTKKMPQVSTDTLLEIGKKMTEVGNKCCQLPQDKRLPCSEGY  480
Anna's Hummingbird       401  AVEELNKHIQETQDVVKTNCELLNTHGEADFLKAILIRYTKKMPQVSTDTLVEIGKKMTAVGTKCCQLPEDRRLPCSEGY  480

Human                    477  LSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADI   CTLSEKERQIKKQ-TA  552
Cow                      476  LSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTFHADI   CTLPDTEKQIKKQ-TA  551
Chicken                  481  LSIVIHDTCRKQETTPINDNVSQCCSSSYANRRPCFTAMGVDTKYVPPPFNPDMFSFDEKL   CSAPAEEREVGQM-KL  556
Golden-collared manakin  481  LSIVIEKMCKKQESTPINDQVTQCCNEFYSYKRPCFTAMGVDTKYVPPPFDPTMFNFDDKL   CSAPPAERREEGQL-KL  556
American crow            481  LSIIIEDMCKRQESTPINDQVSQCCNELYSYRRPCFTAMGVDTKYVPPPFDPMMFNFDEKM   CSAPPAERREEGQL-KL  556
Hoatzin                  481  LSIVIQDMCRRQETTPINDNVSHCCSDSYAYRRPCFTAMGVDTKYVPPPFDPEMFSFDQKL   CTASPAEQEIGNM-KL  556
Killdeer                 481  LSIVIQDMCRRQETTPINDNVSHCCSDSYAYRRPCFTAMGVDTKYVPPAFDPEMFSFDEKL   CTASPAERELGQM-KL  556
S. carmine bee-eater     481  LSIVIHEMCRRQETTPINDNVSHCCSDSYAYRRPCFTAMEADTKYVPPPFDPTMFNFDDKL   CSAPPLTMYCGFVcRL  557
Brown mesite             481  LSIVIQDMCRRQETTPINDNVSHCCSDSYSYRRPCFTAMGVDTKYVPPAFNPEMFSFDEKL[5]CSAPPAEQELGQM-KL  561
Sunbittern               481  LSIVIHDMCRKQETTPINDNVSQCCSNSYAYRRPCFTAMGIDTKYVPPAFNPDMFNFDEKL   CTAAPDEQELGQM-KL  556
Rifleman                 481  LNIVIDDMCKKQESTPVNDQVSHCCSEPYSDRRPCFTTMGVDPKYVPPPFDPQMFNFDDKL   CTAPPAERREEGQL-KL  556
Adelie penguin           481  LSIVMQDMCRRQETTPINDNVSHCCSDSYAYRRPCFTAMGVDTKYVPPAFDPEMFSFDEKL   CTAPPAERELGQM-KL  556
Emperor penguin          481  LSIVIQDMCRRQETTPINDNVSHCCSDSYAYRRPCFTAMGVDTKYVPPAFDPEMFSFDEKL   CTAPPAERELGQM-KL  556
Red-throated loon        481  LSIVIQDMCRRQETTPINDNVSHCCSDSYAYRRPCFTAIGVDTKYVPPAFDPEMFSFDEKL   CTAPPAERELGQM-KL  556
Crested ibis             481  LSIVIQDMCRKQETTPINDNVSHCCDDSYAYRRPCFTAMGVDTKYVPPPFNPDMFNFDEKL   CTASPPEREVGQM-KL  556
Barn owl                 481  LSIVIQDMCRRQETTPINDNVSHCCSDSYAYRRPCFTAMGVDTKYVPPAFDPEMFSFDEKL   CTASPAERDLGHM-KL  556
Anna's Hummingbird       481  LSIVIQDMCRRQETTPINDNVSHCCSDSYSNRRPCFTSMGVDAKYVPPAFNPDMFNFDETL   CTASLAERESGQM-KL  556

Human                    553  LVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL--  609
Cow                      552  LVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEACFAVEGPKLVVSTQTALA---  607
Chicken                  557  LINLIKRKPQMTEEQIKTIADGFTAMVDKCCKQSDINTCFGEEGANLIVQSRATLGIGA  615
Golden-collared manakin  557  LINLIKRKPQMTEDQIKAIAGGFTAMVEKCCKAADVDTCLGEEGAALIVQSRATLGIDA  615
American crow            557  LVNLIKRKPQMTEEQLKTIAGGFTAMMEKCCKQSDIDACLGEEGAALIVQSRATLGIGA  615
Hoatzin                  557  LINLIKRKPQMTEEQIKTIIGGFTAMVDKCCKQADVETCFGEEGANLIVQSRATLGIGA  615
Killdeer                 557  LVNLIKRKPQMTEEQIKTIADGFTAMVDKCCKQSDIETCFGEEGANLIVQSRATLGIGA  615
S. carmine bee-eater     558  LINLIKRKPQMTEEQIKTIADGFNAMVDKCCKESDIDTCFGEEGANLIVQGRAILGIGA  616
Brown mesite             562  LVNLIKRKPQMTEEQITTIATGFTAMVDKCCKQPDIETCFGEEGANLIVQSRATLGIGA  620
Sunbittern               557  LVNLIKRKPQMTEEQIKTIADGFTAMVQKCCKQSDIETCFGEEGAVLIVQGRATLGIGA  615
Rifleman                 557  LVNLIKRKPQMTEEQIKTVAGGFTAMMDKCCKESDIDTCLGEEGANLIVQSRATLGIGA  615
Adelie penguin           557  LVNLIKRKPQMTEEQIKTIADGFTAMVDKCCKQSDIDTCFGEEGANLIVQSRATLGISA  615
Emperor penguin          557  LVNLIKRKPQMTEEQIKTIADGFTAMVDKCCKQSNIDTCFGEEGANLIVQSRAILGISA  615
Red-throated loon        557  LVNLIKRKPQMTEEQIKTIADGFTAMVEKCCKQSDIETCFGEEGANLIVQSRATLGIGA  615
Crested ibis             557  LINLIKRKPQMTEEQIKTIADGFTAMVDKCCKQTDIETCLGEEGANLIVQSRATLGIGA  615
Barn owl                 557  LVNLIKRKPQMTEEQIKTIAEGFTAMVDKCCKQSDIETCFGEEGANLIVQSRATLGIGA  615
Anna's Hummingbird       557  LVNLIKRKPQMTEEQIKTIADGFSAMVDKCCKESDVGSCLGEEGANLIVQSRATLGIGA  615
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